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TITLE: Preclinical Validation of Anti-Nuclear Factor Kappa B Therapy against Vestibular Schwannoma and Neurofibromatosis Type II

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CONTRACTING ORGANIZATION:

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14. ABSTRACT

Background: Neurofibromatosis type 2 (NF2) is a genetic disorder that causes substantial suffering and debility due to many tumors that occur on the nerves within the skull and spine throughout a person's life. The hallmark of NF2 is vestibular schwannomas (VSs), also known as acoustic neuromas, which occur on the vestibular nerves that connect the inner ear with the brain. Initially, VSs cause hearing loss. However, as they grow, they can compress the brainstem and cause death. Current treatment options are limited to surgical removal and radiation therapy, both of which carry substantial risks, including deafness and facial paralysis. Although drug therapies against NF2 are gaining momentum, more effective and better tolerated drugs are sorely needed. Because NF2 tumors are typically slowly growing and non-malignant, even therapies that simply reduce tumor volume and retard growth can be life-saving. The most successful drug used today to treat NF2, bevacizumab, works in only about 50% of patients in halting tumor growth or causing tumor shrinkage. Bevacizumab is known to inhibit vascular endothelial growth factor (VEGF), but its precise mechanism of action in VSs is unknown. During the first year of DoD funding, we demonstrated that a specific inhibition of a pro-inflammatory transcription factor nuclear factor kappa B (NFκB) can be cytotoxic for VS cells *in vitro*. We also demonstrated a cytostatic effect of non-steroidal anti-inflammatory drugs (NSAIDs) on VS cells *in vitro*, suggesting novel treatment options for VS and NF2.

Progress: Because NFkB is activated by tumor necrosis factor alpha (TNFα) signaling, and TNFα is known to be ototoxic, over the last year we focused on quantifying VSs secretion of TNFα. We found that TNFα levels in VS secretions correlated with the severity of VS-induced sensorineural hearing loss. We then demonstrated that TNFα blockade in VS secretions prevented cochlear damage due to application of VS secretions *in vitro*. This suggests that therapeutic blockade of TNFα (and hence of NFkB) may prevent both VS growth and the associated hearing loss. Using human volumetric VS measurements in a retrospective cohort, we have reaffirmed our previous finding that the intake of aspirin (which partly signals via NFkB) correlates with reduced growth of VS. We have also established the *in vivo* murine model with a human VS xenograft for testing of promising therapies.

Planned experiments: With the animal model established, we will focus on experiments *in vivo* to further test the hypothesis that inhibition of the NFκB pathway can prevent growth and promote death of VSs. We will test the efficacy of NFκB inhibition alone or in combination with anti-VEGF therapy as new treatments for NF2. The mice will receive curcumin, or anti-VEGF antibody, or both, or neither. We will monitor tumor growth and response to therapy in alive mice through blood assays and bioluminescence imaging.

Applicability: Our work is applicable for patients with stable or progressive NF2 disease, with a particular focus on VSs, hallmark tumors of NF2. Our work also has implications for the closely related diseases such as schwannomatosis, which sometimes manifests with VS, and sporadic VS, which affects vestibular nerves on one side only. Drug therapies for sporadic VS are virtually non-existent. More broadly, our work may affect treatment of patients with cancers that have increased activity of NFκB and VEGF, such as breast, colon and ovarian cancer.

Contributions – existing and potential: Our results thus far have stimulated the design of a new clinical trial to prevent VS growth with anti-inflammatory medications such as aspirin. If our proposed experiments *in vivo* support our findings *in vitro* that curcumin inhibits VS proliferation, this will stimulate a clinical trial with curcumin because it is extremely well tolerated when taken as a dietary supplement or therapy for several types of disease. By delineating what, if any, fraction of anti-VEGF's beneficial effect is via NFκB signaling, and by testing combination therapy against NFκB and VEGF for human cells, we set the stage for a future clinical trial using combination therapy targeting NFκB and VEGF, a concept that is widely accepted in the context of cancer but has yet to be applied to NF2 treatment.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**	Unclassified	34	**19b. TELEPHONE NUMBER** *(include area code)*
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1. INTRODUCTION

Background: Neurofibromatosis type 2 (NF2) is a genetic disorder that causes substantial suffering and debility. Current treatment options are limited to surgical resection and stereotactic radiation therapy, both of which carry substantial risks, including deafness and facial paralysis. Although pharmacotherapies against NF2 are gaining momentum, the most successful so far, namely a vascular endothelial growth factor (VEGF) inhibitor bevacizumab, halts or retards tumor growth in only 54% of the patients, and can also cause significant side effects such as renal failure. Pharmacologic treatment of NF2 represents a major unmet medical need. The hallmark of NF2 is bilateral vestibular schwannomas (VSs), which arise from the vestibular nerves. Although VSs typically cause hearing loss, they can lead to death from brainstem compression. We focus on VS as a model NF2 tumor to develop well tolerated pharmacotherpies to treat NF2.

Objective/Hypothesis: Using a systems biology-based bioinformatic analysis of all genes implicated in VS pathobiology, we found nuclear factor kappa B (NFκB), a pro-inflammatory transcription factor, to be aberrantly upregulated, translated and activated in VS in comparison to healthy nerves. Although novel in NF2 and VS, NFκB is known to play a major role in various other neoplasms. Our **leading hypothesis** is that upregulation of NFκB signaling and inflammation in VS contributes to neoplastic growth, and that specific targeting of the NFκB pathway and inflammation can be therapeutic against VS. Thus far we have proven the hypothesis *in vitro* (**Aim 1**), using primary human VS cells and the human NF2 VS cell line compared to control primary human Schwann cells. These cells were treated with (1) siRNA, (2) an experimental drug, BAY11, or (3) a dietary supplement, curcumin. We have also discovered an additional therapeutic target in VS: HGF (hepatocyte growth factor)-cMET pathway, which interacts with the VEGF pathway (**Aim 2**). We now plan to focus on testing the leading hypothesis *in vivo*, using human VS xenografts on the sciatic nerve in nude mice (**Aim 3**).

Specific Aims:

    Aim 1: Define parameters for and therapeutic specificity of NFκB inhibition in human VS cells without causing cytotoxicity in healthy human Schwann cells *in vitro*.
- This was completed and two papers were reported as a part of last year's progress report. Two more papers were published and one accepted during this year.

    Aim 2: Determine whether therapeutic VEGF inhibition in VS cells acts solely though the VEGFR pathway *in vitro*.
- This was completed and a paper was published.

    Aim 3: Test efficacy of NFκB inhibition *in vivo* using primary human VS cells xenografted onto the sciatic nerve of nude mice.
- We have established the mouse model and will use it to test our leading hypothesis over the next year.

Innovation: Our focus on NFκB as a potent pharmacologic target for NF2 is novel. Our work emphasizes the role of pathological inflammation in NF2 and VS, an aspect that has not been explored but could be a major modulator of the disease. We use NFkB inhibitors that are novel to the NF2 and VS fields: BAY 11, an experimental drug, and curcumin, a dietary supplement. Our exploration of cocktail phamacotherapy for NF2 is innovative, and has a potential to prevent resistance to and toxicity of bevacizumab through synergy with curcumin. Unlike many pharmacotherapies in clinical trials against NF2 today, curcumin is extremely well tolerated, and effective against several malignancies.

       Furthermore, our discovery that human vestibular schwannoma secretions can cause direct cochlear damage is novel and provides a mechanism for VS-induced sensorineural hearing loss, in addition to physical compression of the auditory nerve. We have provided the first demonstration that TNFα, which triggers NFkB activation, is an ototoxic molecule in VS secretions and that VSs secrete extracellular vesicles that can directly damage cochlear cells. These discoveries provide novel therapeutic targets to treat VS-induced hearing loss, possibly in combination with therapies aimed at preventing VS growth.

2. KEYWORDS (and ABBREVIATIONS)
- 5-ASA: 5-aminosalycilic acid
- BAY11: BAY11-7082)
- cMET: MNNG HOS transforming gene

- COX2: cyclooxygenase 2
- EV: extracellular vesicle
- GAN: Great auricular nerve
- HGF: Hepatocytes growth factor
- IκBα: Inhibitor of kappa B alpha
- MET: gene encoding cMET protein
- NaSal: sodium salicylate
- NIH: National Institutes of Health
- NF2: Neurofibromatosis type 2
- NF-κB: Nuclear factor kappa B
- PTG: prostaglandin
- SC: Schwann cell
- siRNA: small interfering RNA
- S100: Schwann/schannoma cell marker
- TNFα: Tumor necrosis factor alpha
- VEGF: Vascular endothelial growth factor
- VEGFR: vascular endothelial growth factor receptor
- VS: Vestibular schwannoma

3. ACCOMPLISHMENTS

3.1 Activities in relation to statement of work.

Aim 1: Define parameters for and therapeutic specificity of NFκB inhibition in human VS without causing cytotoxicity in healthy human Schwann cells in vitro.

This work was completed and published last year, as summarized in the progress report for 2014-2015.

Because of NFkB's pro-inflammatory role, we also tested (and reported last year) another group of related anti-inflammatory medications in the treatment of VS: salicylates. In line with our published reports on the cytostatic effect of aspirin *in vitro* (Dilwali et al, 2015) and *in vivo* (Kandathil et al. 2014), our most recent retrospective clinical study found a correlation between aspirin intake and reduced growth of VS, based on the volumetric tumor analysis (Kandathil et al, accepted). Our volumetric analysis of VS growth reaffirms the results of our linear analysis and suggests that aspirin may inhibit VS growth. Consequently, we have resubmitted a grant application for a prospective randomized phase 2 clinical trial of aspirin for VS.

Because NFkB is activated by tumor necrosis factor alpha (TNFα) signaling, and TNFα is known to be ototoxic, we further tested whether VSs secreted TNFα. We found that TNFα secretion by VSs correlated with the severity of VS-induced sensorineural hearing loss. We then demonstrated that TNFα blockade in VS secretions can prevent cochlear damage due to application of VS secretions *in vitro*. This suggests that therapeutic blockade of TNFα (and hence of NFkB) may prevent both VS growth and the associated hearing loss. Details are provided in a recently published paper (Dilwali et al, Scientific Reports 2015), as summarized in sections 6.1 and 9 below.

Aim 2: Determine whether therapeutic VEGF inhibition in VS cells acts solely though the VEGFR pathway in vitro.

This work was completed and published last year, as summarized in the progress report for 2014-2015.

Aim 3: Test efficacy of NFκB inhibition in vivo using human VS cells xenografted onto the sciatic nerve of nude mice.

The animal protocol to pursue these experiments has been approved. The animal model is based on xenografting of HEI-193 cells (from an NF2 patient) or primary human VS cells onto the sciatic nerve of nude

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mice. We have treated HEI-193 cell xenografted mice with curcumin and bevacizumab, and have identified 2 important technical issues, and ways to overcome them.

First, intraperitoneal delivery of curcumin dissolved in oil results in abdominal bloating in mice, and consequently unknown levels of systemic absorption. To overcome this issue, the next series of experiments will utilize orally delivered curcumin in mouse chow, as described in a recent publication:
Bimonte S et al. Dissecting the role of curcumin in tumour growth and angiogenesis in mouse model of human breast cancer. Biomed Res Int. 2015;2015:878134.
In addition to monitoring dietary uptake of color-coded chow with curcrmin, we also plan to measure curcumin levels in serum. We expect that these modifications will enable acquisition of robust and interpretable data.

Second, bevacizumab is not effective when given to mice. This is likely due to the species difference because bevacizumab is a humanized anti-VEGF monoclonal antibody. We therefore plan to use a mouse anti-VEGF antibody instead of bevacizumab in animal experiments planned for the next year. Our collaborator and neighbor, Dr. Lei Xu at Massachusetts General Hospital, has recently published the dosing protocol for an effective anti-VEGF antibody treatment in mice:
Gao X et al. Anti-VEGF treatment improves neurological function and augments radiation response in NF2 schwannoma model. Proc Natl Acad Sci U S A. 2015 Nov 24;112(47):14676-81.
We expect that this simple change will allow us to complete Aim 3 in the course of the next year.

3.2. Opportunities for professional development:

I have deepened my professional connections with thought leaders in neurofibromatosis research by attending and presenting at multiple meetings, including local (at Massachusetts General Hospital, Massachusetts Eye and Ear, Radcliffe Institute), national (at the University of South Carolina, University of Miami, annual meeting of the American Academy of Otolaryngology, annual meeting of the American College of Surgeons, annual meeting of the Associating for Research in Otolaryngology) and international (in Medelline, Colombia) meetings (summarized in **section 6.2**). I also attended the NF2 State of the Art meeting in New York in March 2016 where I had a chance to interact with national and international NF2 researchers. I am now a formal member of the Harvard-wide group of NF2 investigators who work jointly to overcome the silo mentality and accelerate development of cures for NF2.

I have had an opportunity to make our research accessible to NF2 patients and invite their input by giving an invited talk at a meeting of the Acoustic Neuroma Association (ANA) in Boston. The talk was well received and is archived at a part of the ANA video library.

3.3. Dissemination of Results

Results have been disseminated by publications in scientific journals, by presentations at scientific meetings, by presentations at patient advocacy groups (Acoustic Neuroma Association) and policy makers (at a Leadership Forum at Massachusetts Eye and Ear), and by media via websites (detailed in Section 6).

3.4 Plans for the next reporting period

The focus for the next year will be testing the efficacy of combination therapy targeting NFκB (with curucmin) alongside targeting VEGF with mouse anti-VEGF antibody *in vivo* (**Aim 3**). Xenografted mice will consume oral curcmin or will be injected with mouse anti-VEGF antibody, or will receive combination therapy, or will receive vehicle alone. NFkB activity and tumor growth will be tracked longitudinally, utilizing Gluc-based blood assays and Fluc-based bioluminescence imaging, respectively. Histological studies will define tumor response and systemic toxicity *post mortem*.

4. IMPACT

Our studies address NFRP's area of emphasis on drug discovery for the treatment of NF2. Our preclinical studies pave the way for rapid translation to clinical trials because we study NFκB inhibitors that include a dietary supplement, curcumin, alone or in combination with anti-VEGF therapy; bevacizumab (a humanized anti-VEGF antibody) is already used in NF2 patients. Importantly, inhibiting the NFκB pathway may be important not only for preventing tumor growth but also for preventing the associated hearing loss, because we find that VS secretion of TNFα (which is an activator of NFκB) correlates with the severity of hearing loss. By studying primary VSs, in addition to the NF2 VS cell line, we explore heterogeneity of NF2-related tumors, which is NFRP's area of emphasis. More broadly, our studies have a potential to impact treatment of related diseases, sporadic VS and schwannomatosis, as well as many human malignancies that are characterized by dysregulated NFκB and VEGF signaling.

5. CHANGES/PROBLEMS

None

6. PRODUCTS

6.1. Published and accepted manuscripts:

1. Dilwali KS, Landegger LD, Soares VYR, Deschler DG, **Stankovic KM**. Secreted factors from human vestibular schwannomas can cause cochlear damage. *Scientific Reports* 2015; 5:18599. Acknowledgement of federal support: yes.
   - Paper was featured on Medscape Oncology web site and Harvard Medical School web site.
2. Soares VYR, Atai NA, Fujita T, Dilwali S, Sivaraman S, Landegger LD, Hochberg FH, Oliveira CAPC, Bahmad F, Breakefield XO, **Stankovic KM**. Extracellular vesicles derived from human vestibular schwannomas associated with poor hearing damage cochlear cells. *Neuro-Oncology* 2016. May 18. pii: now099. [Epub ahead of print]. Acknowledgement of federal support: yes.
3. Kandathil, C. Cunnane ME, Curtin H, **Stankovic KM**. Correlation between aspirin intake and reduced growth of human vestibular schwannoma: volumetric analysis. *Otology and Neurotology*. Accepted. Acknowledgement of federal support: yes.

6.2. Oral presentations:

1. **Stankovic KM**: Molecules that control human hearing: insights from vestibular schwannoma, *Invited Otolaryngology Research Seminar, Medical University of South Carolina, Charleston, SC in August 2015*.
2. **Stankovic KM**: Update on model systems for NF2. *Invited presentation at the symposium "Escaping the silo mentality: creating a joint vision for gene therapy in NF2", Radcliffe Institute for Advanced Study, Harvard University, Cambridge, MA in September 2016*.
3. **Stankovic KM**: Choosing model systems for preclinical development of gene therapy and deciding what would constitute a "go" signal as preclinical efficacy to move to clinical trial. *Roundtable discussant at the symposium "Escaping the silo mentality: creating a joint vision for gene therapy in NF2", Radcliffe Institute for Advanced Study, Harvard University, Cambridge, MA in September 2015*.
4. **Stankovic KM:** Modern otology: a nexus of surgery, neuroscience and biotechnology. *Invited Howard P. House, MD Memorial Lecture for Advances in Otology, American Academy of Otolaryngology – Head and Neck Surgery in Dallas, TX in September 2015*. The talk highlighted our research on vestibular schwannoma and NF2.
   - Highlights of the presentation featured in ENTtoday, a publication of the Triological Society.
5. **Stankovic KM**: Secreted factors from human vestibular schwannomas can cause cochlear damage. *Invited Oral Abstract, Satellite Meeting of the American Academy of Otolaryngology – Head and Neck Surgery and the Middle Eastern Otorhinolaryngology Societies in Dallas, TX in September 2015*.

6. **Stankovic KM**: Secreted molecules that control human hearing. *Invited grand rounds, Massachusetts Eye and Ear in Boston, MA in November 2015*.
7. **Stankovic KM**: Vestibular schwannomas: mechanisms of tumor growth and hearing loss. *Invited Presentation, Center for Human Genetic Research, Massachusetts General Hospital, Boston, MA in December, 2015.*
8. **Stankovic KM**: Exploring novel therapies for vestibular schwannoma. *Invited Presentation, Acoustic Neuroma Association, Boston, MA in December, 2015.*
   - Talk archived on Acoustic Neuroma Association web site
9. Landegger et al **(PI: Stankovic KM)**: Secreted factors from human vestibular schwannomas can cause cochlear damage. *Selected oral abstract, Association for Research in Otolaryngology meeting in San Diego, CA in Feb 2016.*
10. **Stankovic KM**: Secreted factor that control human hearing: insights from vestibular schwannoma. *Invited Presentation, Massachusetts Eye and Ear Leadership Forum, Boston, MA in April 2016.*
11. **Stankovic KM**: Molecular mechanism of hearing loss due to vestibular schwannoma: implication for diagnosis and treatment.*Invited Visiting Professor talk, VII International Otology and Neurotology Meeting, Medellin, Colombia.*
12. **Stankovic KM** : Otology and Neurotology: from bedside to bench and back. *Chandler Visiting Professor and Distinguished Guest Judge, annual Resident/Fellow Research Presentation Day, Department of Otolaryngology, University of Miami Miller School of Medicine in June 2016*. The talk highlighted our research on vestibular schwannomas and NF2.
13. **Stankovic KM**: Extracellular vesicles derived from human vestibular schwannomas associated with poor hearing damage cochlear cells. *Selected oral abstract, triennial meeting of the International Otopathology Society. Boston, MA in June 2016.*

6.3. Posters:

1. Landegger LD, Dilwali KS, Soares VYR, Deschler DG, **Stankovic KM**. Secreted factors from human vestibular schwannomas can cause cochlear damage. *Presented at the American College of Surgeons, Massachusetts Chapter 62nd Annual Meeting in Boston, MD in December 2015 and at the 13th Annual Celebration of Clinical Research at Massachusetts General Hospital.*

6.4 Websites that disseminated the results of the research activities:

Talk on the potential role of aspirin in inhibiting vestibular schwannoma growth highlighted on Acoustic Neuroma Association (ANA) web site and archived in video library:

- ANA, November 2015: https://www.anausa.org/finding-support-overview/support-groups/ana-lsgm-video-library

Press releases about secreted factors from human vestibular schwannomas that can cause cochlear damage:

- Massachusetts Eye and Ear news, December 22, 2015: http://www.masseyeandear.org/news/press-releases/2015/12/toxic-secretions-from-intracranial-tumor-damage-the-inner-ear
- Harvard Medical school, December 23, 2015: https://hms.harvard.edu/news/new-mechanism
- Medscape Oncology, December 28, 2015: http://www.medscape.com/viewarticle/856560
- Harvard Otolaryngology newsletter, Spring 2016 (page 27): https://issuu.com/hmsotolaryngology/docs/harvardotolarynmagazine-singlepgs_bb2673ff58f229

Press releases about Howard House MD Memorial Lecture at the Annual Meeting of the American Academy of Otolarygnology in Dallas, TX (the lecture included highlights of our recent work on vestibular schwannomas):

- ENTtoday, December, 2015: http://www.enttoday.org/article/neuroscience-biotechnology-combination-used-to-tackle-inner-ear-problems/

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name:	*Konstantina Stankovic, MD, PhD*
Project Role:	*PI*
Nearest person month worked:	5.64
Contribution to Project:	*Dr. Stankovic helped with collection of clinical specimens, she designed experiments, oversaw proper data collection and analyses. She wrote papers and presented at scientific meetings.*

Name:	*Vitor YR Soares, MD*
Project Role:	*Post-doctoral fellow and graduate student*
Nearest person month worked:	*6*
Contribution to Project:	*Dr. Soares performed tissue culture experiments*
Funding Support:	*University of Brasilia, Brasilia, Brazil*

Name:	*Takeshi Fujita, MD, PhD*
Project Role:	*Post-doctoral fellow*
Nearest person month worked:	*8*
Contribution to Project:	*Dr. Fujita helped with the collection of clinical specimens, cell culture experiments and animal experiments in vivo.*
Funding Support:	*Kobe University Graduate School of Medicine, Japan*

Name:	*Sonam Dilwali*
Project Role:	*Graduate Student*
Nearest person month worked:	*1*
Contribution to Project:	*She performed tissue culture experiments and helped draft the manuscripts.*
Funding Support:	*T32 DC00038*

Name:	*Lukas Landegger*
Project Role:	*Graduate Student*
Nearest person month worked:	*1*
Contribution to Project:	*Lukas Landegger helped with collection of clinical specimens and tissue culture experiments*
Funding Support:	*Nancy Sayles Day Foundation*

7.2 Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report.

7.3. Other organizations involved as partners:

    Nothing to report

8. SPECIAL REPORTING REQUIREMENTS
None

9. APPENDICES

9.1.   Published paper 1:

Dilwali KS, Landegger LD, Soares VYR, Deschler DG, **Stankovic KM**. Secreted factors from human vestibular schwannomas can cause cochlear damage. *Scientific Reports* 2015; 5:18599. Acknowledgement of federal support: yes.

9.2.   Published paper 2:

Soares VYR, Atai NA, Fujita T, Dilwali S, Sivaraman S, Landegger LD, Hochberg FH, Oliveira CAPC, Bahmad F, Breakefield XO, **Stankovic KM**. Extracellular vesicles derived from human vestibular schwannomas associated with poor hearing damage cochlear cells. *Neuro-Oncology* 2016. May 18. pii: now099. [Epub ahead of print]. Acknowledgement of federal support: yes.

9.3.   Accepted paper 3:

Kandathil, C. Cunnane ME, Curtin H, **Stankovic KM**. Correlation between aspirin intake and reduced growth of human vestibular schwannoma: volumetric analysis. *Otology and Neurotology*. Accepted. Acknowledgement of federal support: yes.

OPEN

Secreted Factors from Human Vestibular Schwannomas Can Cause Cochlear Damage

Sonam Dilwali[1,2,3,*], Lukas D. Landegger[1,4,5,*], Vitor Y.R. Soares[1,5,6], Daniel G. Deschler[2,5] & Konstantina M. Stankovic[1,2,3,5,†]

Vestibular schwannomas (VSs) are the most common tumours of the cerebellopontine angle. Ninety-five percent of people with VS present with sensorineural hearing loss (SNHL); the mechanism of this SNHL is currently unknown. To establish the first model to study the role of VS-secreted factors in causing SNHL, murine cochlear explant cultures were treated with human tumour secretions from thirteen different unilateral, sporadic VSs of subjects demonstrating varied degrees of ipsilateral SNHL. The extent of cochlear explant damage due to secretion application roughly correlated with the subjects' degree of SNHL. Secretions from tumours associated with most substantial SNHL resulted in most significant hair cell loss and neuronal fibre disorganization. Secretions from VSs associated with good hearing or from healthy human nerves led to either no effect or solely fibre disorganization. Our results are the first to demonstrate that secreted factors from VSs can lead to cochlear damage. Further, we identified tumour necrosis factor alpha (TNFα) as an ototoxic molecule and fibroblast growth factor 2 (FGF2) as an otoprotective molecule in VS secretions. Antibody-mediated TNFα neutralization in VS secretions partially prevented hair cell loss due to the secretions. Taken together, we have identified a new mechanism responsible for SNHL due to VSs.

Vestibular schwannomas (VSs) are the most common tumours of the cerebellopontine angle and the fourth most common intracranial neoplasms. Although VSs arise from the vestibular nerves, 95% of patients with VS present with sensorineural hearing loss (SNHL). The underlying pathophysiological mechanisms of this SNHL are currently unknown[1].

Vestibular schwannomas occur sporadically or in association with neurofibromatosis type II (NF2), a debilitating disease whose hallmark is bilateral VSs. In addition to SNHL, VSs can cause facial paralysis, disequilibrium, other cranial neuropathies and even death from brainstem compression[2].

Currently, there are no FDA approved drugs to prevent or treat VS or the associated SNHL. However, two classes of drugs have demonstrated some efficacy in ameliorating SNHL due to VS via unknown mechanisms: bevacizumab, a monoclonal antibody against vascular endothelial growth factor A (VEGF-A) improves hearing in 54% of NF2-associated VS[3], whereas corticosteroids can improve sudden SNHL associated with sporadic and NF2-associated VS[4]. These clinical clues and the unmet medical need to prevent and treat VS-associated SNHL motivate our work in understanding the mechanism of SNHL due to VS.

The predominant hypothesis has been that VSs cause SNHL by mechanically compressing the adjacent auditory nerve. However, this hypothesis does not explain the lack of correlation between the radiographic tumour size or tumour extent within the internal auditory canal and audiometric threshold shifts in people with sporadic VS[5,6]. Further, some patients develop audiometric threshold shifts despite the lack of VS growth[6].

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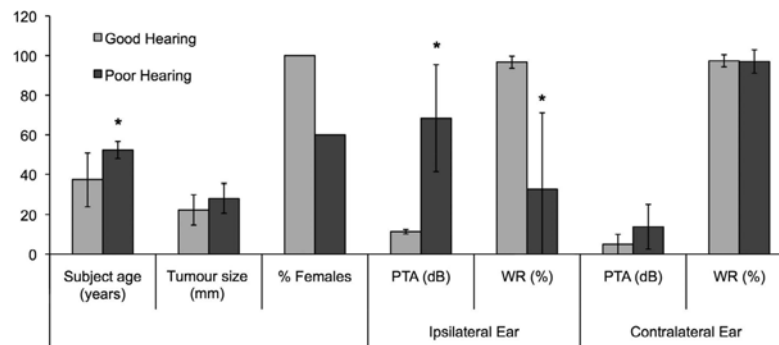
Figure 1. Patient demographics for VS secretions applied to cochlear explants. Average subject age in years, tumour size (mm, largest transverse dimension), % females, pure tone average (PTA, dB), and word recognition score (WR, %) are given for the ipsilateral and contralateral ears to VS. Data are segregated into groups of VS associated with good hearing (PTA < 30 dB and WR > 70%, n = 3 being VS2, VS5 and VS9) and poor hearing (n = 10). * = p < 0.05.

The loss or damage of structures within the inner ear due to VS has been implicated in previous work. Sound-induced vibration of fluids within the inner ear leads to stimulation of cochlear sensory hair cells and excitation of the auditory nerve, which induces activity in more central auditory centres. In addition to behavioural threshold audiometry, there are two commonly used physiologic metrics for evaluation of auditory function: distortion-product otoacoustic emissions (DPOAEs), which are generated by cochlear outer hair cells (OHCs); and auditory brainstem evoked response (ABR), which is a surface potential consisting of multiple waves, with wave I representing the summed activity of the cochlear nerve, and later waves representing contributions from ascending auditory nuclei in the brainstem. Although VSs lead to delayed propagation and decreased amplitude of ABR neural impulses[7,8], they also reduce DPOAE amplitudes, consistent with cochlear dysfunction. Importantly, decreased DPOAEs are present in VS patients with mild SNHL[9], suggesting that OHC dysfunction could be primary, happening early in the progression of the SNHL, rather than secondary to auditory nerve fibres or neurons. These physiologic findings *in vivo* are corroborated by *post mortem* histopathological analyses of temporal bones of individuals with untreated VS-specifically, substantial ipsilateral cochlear atrophy, including degeneration of organ of Corti that comprises sensory hair cells, loss of spiral ganglion neurons, and atrophy of the stria vascularis[10]. However, no published work has elucidated whether the cochlear, arising within the inner ear, or retrocochlear dysfunction, originating centrally to the inner ear, precedes the other.

An alternative to mechanical compression of the auditory nerve leading to SNHL due to VS is the hypothesis, initially explored by our laboratory, that there are biological differences between VSs that cause SNHL and those that do not[11]. Using cDNA microarrays, we found that VS stratified by hearing can have different gene expression profiles, suggesting that diverging concentrations of potentially ototoxic or otoprotective molecules may contribute to the degree of SNHL seen in VS patients[11]. A subsequent cDNA microarray study discovered that PDGF-A gene expression levels inversely correlated with SNHL in VS patients[12], thus supporting our view that intrinsic genetic differences in VSs could contribute to SNHL.

Radiologic studies have suggested that differences in cochlear fluids contribute to SNHL associated with VS. Specifically, the differential intensity of fluid-attenuated inversion recovery magnetic resonance imaging (MRI) sequences from the cochlea, most likely representing the protein density in cochlear fluids, correlates with the degree of SNHL due to NF2-associated and sporadic VSs[13–15]. Importantly, unlike sporadic VS, NF2-associated VS size correlates with the degree of SNHL, suggesting that mechanical compression may be an important factor in SNHL due to NF2 VSs[16], which is why our work focuses on sporadic VS-associated SNHL.

We explore potential ototoxic or neurotoxic biological secretions from VSs that could reach the cochlea via the fundus of the internal auditory canal so to alter cochlear function and hearing. We have previously shown that the proteome of cochlear fluid is different in patients with VS versus without VS[17], suggesting a role of VS-secreted factors in cochlear cell health. Our proteomic study was motivated by earlier reports that total levels of protein in perilymph are 5–15 times higher than in healthy individuals, a difference that was used to diagnose VS prior to the advent of MRI[13,14]. However, no published work thus far has shown a direct effect of VS-associated molecules in causing inner ear degeneration.

Here, we assessed damage in mouse cochlear explants subjected to human VS secretions. Based on the results in the histopathological study of human temporal bones affected by VS[10], we focused on hair cell and neurite loss, while testing VS secretions obtained from subjects with different degrees of ipsilateral SNHL. Our findings suggest that there is a correlation between degree of SNHL in human VS patients and damage caused by the application of tumour secretions on murine cochlear explants. This is the first demonstration that the VS-secreted factors can directly lead to cochlear damage. We have further identified correlations between two secreted molecules and the degree of SNHL, with tumour necrosis factor alpha (TNFα) demonstrating an ototoxic and fibroblast growth factor 2 (FGF2) an otoprotective potential.

Results

Patient Demographics. Demographics of patients with unilateral, sporadic VSs whose tumours were utilized to collect secretions are summarized in Fig. 1 and Table 1. The contralateral ear did not have significant SNHL in any

Tumour ID	Subject Age	Gender	Tumour Size (mm)	Ipsilateral Ear		Contralateral Ear	
				PTA (dB)	WR (%)	PTA (dB)	WR (%)
VS 1	50	F	28	N/A	60	0	100
VS 3	55	F	25	40	20	0	100
VS 4	57	F	21	40	100	15	100
VS 6*	51	F	23	40	92	12	100
VS 7	51	M	25	100	0	35	84
VS 8*	51	M	42	100	0	10	100
VS 10	45	M	39	105	0	5	98
VS 11	48	F	19	65	34	18	100
VS 12	58	F	30	60	8	15	100
VS 13	57	M	29	65	14	28	88
VS 2	47	F	24	12	94	10	100
VS 5	22	F	29	12	96	0	94
VS 9	43	F	14	10	100	5	98

Table 1. Subject demographics for VS secretions applied to cochlear explants for 13 tumours. Subject age, gender, tumour size (mm, largest transverse dimension), pure tone average (PTA, dB), and word recognition score (WR, %) are given for the ipsilateral and contralateral ears to VS. The rows shaded in grey are for VSs associated with GH. N/A = not available. *In an alternate series of experiments, these tumour secretions were subjected to TNF neutralization prior to application.

of the patients except for VS7 and VS13. Out of the thirteen patients, three had good hearing (GH) in the ipsilateral ear (VS2, VS5 and VS9). Age of subject (p = 0.007) and ipsilateral pure tone average (PTA) (p = 0.005) were significantly greater, whereas ipsilateral word recognition score (WR) (p = 0.017) was significantly lower in poor hearing subjects.

Loss of hair cells and neurites in cochlear explants due to vestibular schwannoma secretions.
Application of VS secretions, collected by incubating fresh sporadic VS specimens in culture medium for 72 hours, onto neonatal murine cochlear explants led to variable types and degree of damage in the cochlear cells. Control healthy nerve secretions were collected from surgically sacrificed healthy human great auricular nerves (GANs) in the same manner. We hypothesized that VS secretions from tumours associated with GH or control GAN secretions would not lead to significant cochlear damage, whereas VS secretions from tumours associated with poor hearing (PH) would cause cochlear damage. As anticipated, no hair cell or neurite loss was noted due to secretions from three control GANs or VSs associated with GH (VS2, VS5 and VS9) when specific types of cochlear cells were counted along a 100 μm length in confocal microscopy images. The only significant morphological change was disorganization of the nerve fibres, assessed as a qualitative measure with 0 being highly organized and 2 being severely disorganized, for secretions from two GH tumours (VS2 and VS5) and from one GAN (GAN1). Further, VS secretions from tumours associated with PH (VS1, VS3, VS4, VS6-VS8 and VS10-13) led to a much greater degree of damage in the cochlear explants, exhibiting significant loss of IHCs or OHCs, or fibre disorganization. Apical and basal turns of the murine cochleae were cultured as separate cochlear explants; a greater degree of damage was noted in the basal turns overall. Representative projection images are shown for cochlear explants treated with control media (no treatment (NT)), VS8, VS7, VS6 and VS2 secretions in Fig. 2A (apical turn a–e, basal turn g–k), respectively. Representative images are also shown for cochlear explants treated with GAN secretions (GAN2) in Fig. 2A (apical turn f, basal turn l).

Quantification of confocal images is summarized as average ± standard deviation (SD). N represents the number of different cochlear explant cultures tested for a given treatment. Benjamini-Hochberg adjusted t-test p-values are reported. Data from the cochlear apex and base are analyzed separately because they correspond to different frequency regions. IHC counts per 100 μm length showed VS8 secretions (from a tumour associated with anacusis) leading to significant IHC loss at the apical and basal turns; number of IHCs decreased from 12.3 ± 1.4 (n = 28) for NT to 7.2 ± 5.0 (n = 5, p = 0.0005) with treatment in the apex, and from 13.9 ± 1.5 (n = 26) for NT to 4.3 ± 4.2 (n = 6, p = 10^{-9}) with treatment in the base (Fig. 2B). Additionally, treatment of explants with VS10 secretions (another tumour associated with anacusis) led to significant IHC loss at the basal turn; number of IHCs decreased to 10 ± 1.7 (n = 3, p = 0.002). Treatment of explants with control GAN secretions did not significantly change IHC counts in the apex or base (Supplementary Fig. 1). N is the same in the rest of analyses as for IHC counts for all treatments.

Four of the ten poor hearing tumours caused OHC loss. VS8, VS10 and VS11 secretions led to significant OHC loss in the apical turn: the number of OHCs per 100 μm length decreased from 38.1 ± 4.8 for NT to 23.6 ± 12.1 (p = 0.0004) for VS8, to 25.7 ± 5.1 (p = 0.001) for VS10 and 30.0 ± 2.0 (p = 0.03) for VS11 (Fig. 2C). For the basal turn, VS6 and VS8 secretions led to significant OHC loss, decreasing from 39.8 ± 5.6 for NT to 25.7 ± 3.1 (p = 0.001) and 16.3 ± 6.9 (p = 10^{-8}), respectively (Fig. 2C). Application of control GAN secretions did not significantly change OHC counts in the apex or base (Supplementary Fig. 1).

Although VS9, VS10 and VS11 secretions showed a trend toward reduced neurites with application, the trends did not meet significance (Fig. 2D). The number of neurites per 100 μm length did not significantly change in the apical or basal turns after application of control GAN secretions (Supplementary Fig. 1).
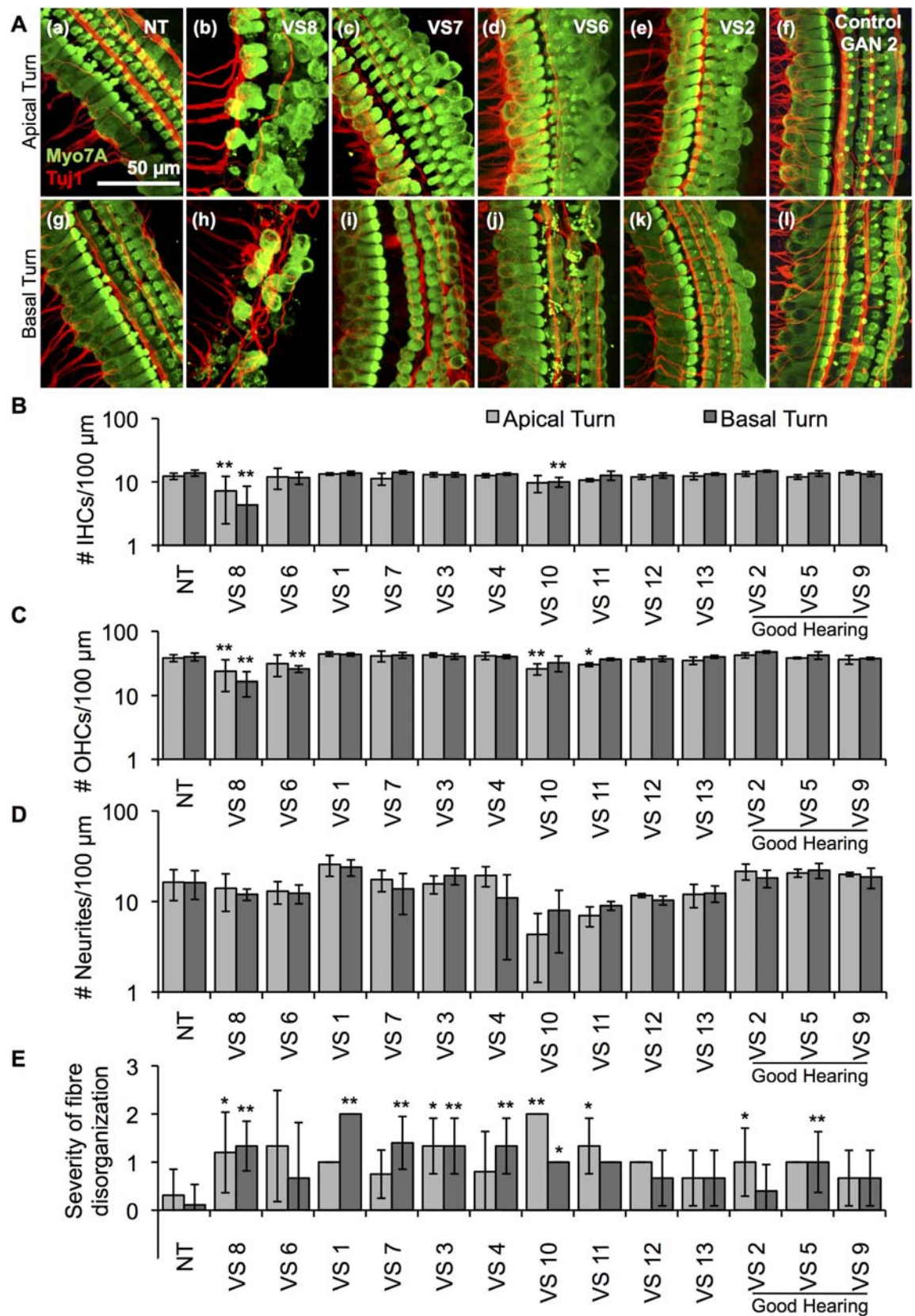
Figure 2. Application of human VS secretions onto murine cochlear explant cultures leads to hair cell and neurite loss. (A). Representative images for cochlear explants receiving (a) no treatment (NT, n = 28 different explants), incubated with (b) VS8 (n = 5 different explants), (c) VS7 (n = 4 different explants), (d) VS6 (n = 3 different explants), (e) VS2 (n = 5 different explants) and (f) control GAN2 (n = 3 different explants) secretions are shown for the apical turns, and (g) NT (n = 26 different explants), (h) VS8 (n = 6 different explants), (i) VS7 (n = 5 different explants), (j) VS6 (n = 3 different explants), (k) VS2 (n = 5 different explants) and (l) control

GAN2 (n = 4 different explants) secretions for the basal turn. Myo7A (green) marks hair cells and Tuj1 (red) marks neurites. Scale Bar = 50 μm applies to all images. (**B**). Number of inner hair cells (IHCs), (**C**). outer hair cells (OHCs), (**D**). neurites, and (**E**). severity of fibre disorganization are shown for a 100 μm length within the apex (light grey columns) and basal turn (dark grey columns) cochlear explants treated with NT and secretions from 13 different tumours. *p < 0.05, **p < 0.01. Quantified data after treatment with control GAN secretions are shown in Supplementary Figure 1.

The severity of nerve fibre disorganization was assessed qualitatively with 0 standing for essentially intact and 2 being most severe. The radial fibres arising from the modiolar region consist of the majority of afferents from the IHCs, with the rest being efferents. The spiraling fibres in the periphery of the cochlear explant are thought to mostly contain efferents coming from the medial olivary complex within the superior olive that synapse onto OHCs to modulate their function as amplifiers, with a smaller population being afferents[18]. Although more proximal centers, such as the superior olive, are not present in the cochlear explant cultures, nerve fibres synapsing at the IHCs and OHCs remained intact and organized in control specimens (Fig. 2A(a)).

Interestingly, nerve fibre disorganization was noted in explants treated with secretions. In the apical turn, secretions from VS2, VS3, VS8, VS10 and VS11 led to significant fibre disorganization, all tumours being associated with PH except VS2. Specifically, severity of fibre disorganization per 100 μm length for the apical turn decreased from 0.3 ± 0.5 for NT group to 1.3 ± 0.6 (p = 0.01), 1.2 ± 0.8 (p = 0.01), 2.0 ± 0.0 (p = 0.008) and 1.3 ± 0.6 (p = 0.03) for explants treated with VS3, VS8, VS10 and VS11 secretions, respectively (Fig. 2E). For VS2, level of fibre disorganization was 1.0 ± 0.7 (p = 0.04), the least drastic change out of all the significant changes. In the basal turn, secretions from VS1, VS3, VS4, VS5, VS7, VS8 and VS10 led to significantly increased fibre disorganization; only VS5 is associated with GH. The values were 0.1 ± 0.5 for NT, 2.0 ± 0.0 (p = 10^{-8}) for VS1, 1.3 ± 0.6 (p = 0.004) for VS3, 1.3 ± 0.6 (p = 0.0004) for VS4, 1.0 ± 0.6 (p = 0.0008) for VS5, 1.4 ± 0.5 (p = 10^{-5}) for VS7, 1.3 ± 0.5 (p = 10^{-5}) for VS8, and 1.0 ± 0.0 (p = 0.05) for VS10 (Fig. 2E). The severity of neurite fibre disorganization was not affected by GAN secretions, except for secretions from GAN1 in the apical turn only, increasing from 0.1 ± 0.4 for NT to 1.0 ± 0.0 (p = 0.04) with GAN1 secretion treatment (Supplementary Fig. 1).

Overall, differential levels of damage were noted due to different VS secretions. There was a general trend of increasing damage from apical to basal turns. Osmolality for the randomly selected tumour secretions did not significantly deviate from control media, being 323, 316 and 324 mOsm/kg for control media, VS7 and VS8 secretions, respectively. The values were near the expected value of 335 mOsm/kg in perilymph for mammals[19].

TNFα correlates directly and FGF2 correlates inversely with degree of SNHL due to VS.   TNFα concentrations were measured in nine tumour secretions, collected in phosphate buffered saline (PBS), using ELISA. These sporadic VS secretions had to be obtained from different tumours than the VS secretions used for the explant experiments described above because of the small specimen size. We focused on TNFα because it is a pro-inflammatory molecule involved in many diseases, including SNHL[20], and the therapeutic effect of anti-inflammatory corticosteroids in treating sudden SNHL due to VS suggests a pathogenic role of inflammatory molecules in SNHL due to VS. The patients whose VS secretions were used had varied levels of ipsilateral SNHL, with pure tone average ranging from 20–100 dB and word recognition score ranging from 0–92%. The contralateral ears for all patients had normal hearing. TNFα levels, on average being 11.0 pg/mL, had a range of 0.0 to 44.2 pg/mL. TNFα levels in the secretions correlated positively and significantly with the subjects' pure tone averages (rho = 0.90, p = 0.0009, Fig. 3A), and correlated negatively with the word recognition scores (rho = −0.71, p = 0.03, Fig. 3B) in the ipsilateral ear.

Reanalyzing cytokine array data that we have previously published[21] for FGF2 levels in 21 VS secretions collected in PBS, FGF2 expression correlated negatively with the pure tone average (rho = −0.43, p = 0.05, Fig. 3C), and correlated positively with word recognition score (rho = 0.47, p = 0.03, Fig. 3D) in the ipsilateral ear as the VS.

Tumour necrosis factor alpha application alone leads to loss and disorganization of neurites in basal turn of cochlear explants.   To understand if tumour necrosis factor alpha (TNFα) has the potential of causing SNHL, we applied recombinant TNFα onto cochlear explant cultures. Cell counts and morphology of recombinant human TNFα-treated cochlear explants were compared with those of age-matched control specimens receiving only PBS (NT). Specific damage in terms of neurite counts and fibre organization of the treated samples was detected in the basal turn. Representative images for NT and TNFα-treated cultures are shown in Fig. 4A (apical turn a–b, basal turn c–d), respectively. Data are summarized as average ± SD for NT and TNFα-treated cochlear explants. N represents the number of different cochlear explant cultures tested for a given treatment. The number of IHCs (Fig. 4B) or OHCs (Fig. 4C) per 100 μm length along the cochlea did not change in the apical or basal turn. While the number of OHCs tended to decrease in the basal turn, the change did not meet significance. Although the neurite counts did not change significantly in the apical turn, they did change significantly in the basal turn, going from 14.8 ± 2.8 for NT (n = 6) to 11.3 ± 2.4 with treatment (n = 6, p = 0.04) (Fig. 4D). The severity of the fibre disorganization, assessed qualitatively with 0 being essentially intact and 2 being most severe, did not change significantly in the apical turn, but did change significantly in the basal turn, going from 0.2 ± 0.4 for NT (n = 6) to 0.8 ± 0.4 with treatment (n = 6, p = 0.02) (Fig. 4E). Osmolality did not deviate from control media, being 330 and 329 mOSm/kg for control media and media with recombinant TNFα, respectively.

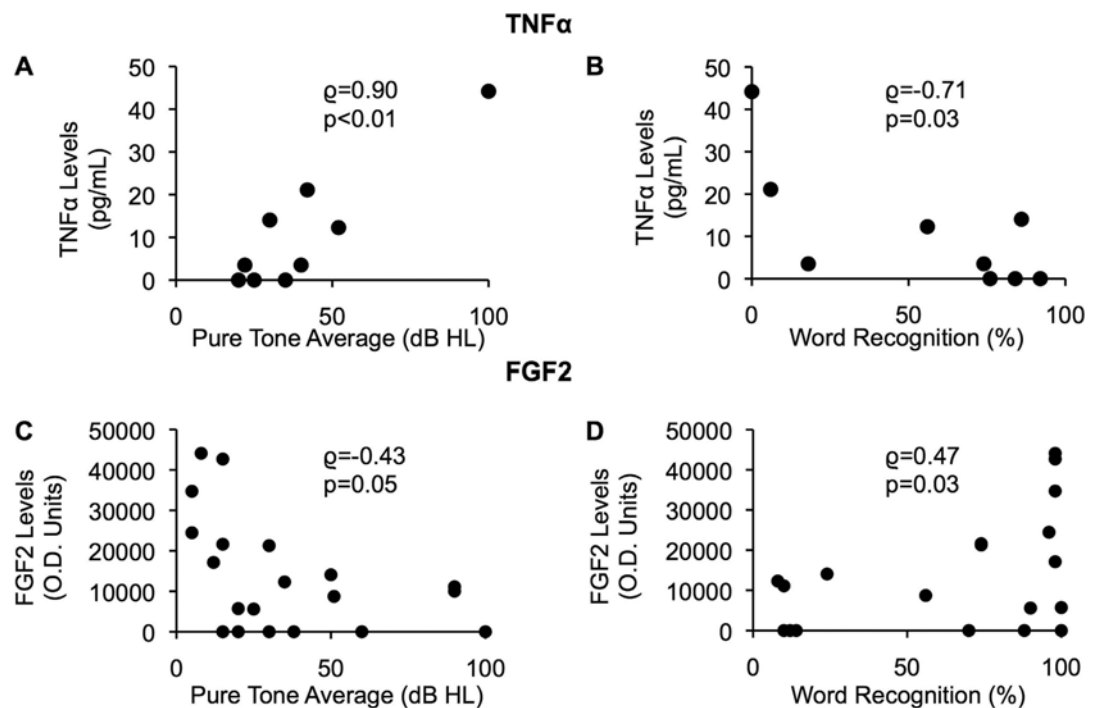**Figure 3. Correlation of VS-secreted TNFα and FGF2 with SNHL due to VS. (A)**. Correlation of pure tone average (dB) and (**B**). word recognition score (%) of ipsilateral ear to the VS, and measured secreted TNFα levels from VSs (n = 9). (**C**). Correlation of pure tone average (dB) and (**D**). word recognition score (%) of ipsilateral ear to the VS and measured secreted FGF2 levels from VSs (n = 21). p-values are shown, rho represents Spearman's rank correlation coefficient.

TNFα neutralization in VS secretions partially prevents hair cell loss due to VS secretions in cochlear explants. In order to more directly assess TNFα's role in VS secretions leading to cochlear damage, secretions from two tumours that caused significant cochlear explant damage and secreted substantial levels of TNFα based on ELISA, namely VS6 and VS8, were incubated with a goat anti-human TNFα antibody prior to application onto cochlear explants. After 2 h of incubation of the secretions with a TNFα antibody or control goat IgG antibody, the secretions, along with antibody, were applied to the cochlear explants for 48 h following the same protocol as for applying secretions alone. Antibody-mediated TNFα neutralization was successful in reducing TNFα levels to 3.7 pg/mL from 17.6 pg/mL in VS6 secretions as measured using ELISA. Interestingly, TNFα neutralization could prevent hair cell loss for VS6 and VS8 (Fig. 4 (F–J)). Specifically, there was significant loss of IHCs due to VS8 in both apical and basal turns, decreasing IHCs from 12.5 ± 1.8 (n = 6) and 13.6 ± 1.5 (n = 5) for NT to 7.3 ± 1.5 (p = 0.03, n = 3) and 6.0 ± 4.2 (p = 0.03, n = 4) for apical and basal turns, respectively (Fig. 4G). TNFα neutralization could significantly prevent the apical IHC loss, increasing the number of hair cells per 100 μm to 14.0 ± 0.0 (p = 0.02, n = 2) (Fig. 4G). Further, OHC loss caused by VS6 in the basal turn, decreasing from 40.4 ± 4.7 (n = 5) to 21.6 ± 5.9 (p = 0.03, n = 5) could be prevented by TNFα neutralization, increasing the number of hair cells per 100 μm to 36.0 ± 5.6 (p = 0.03, n = 4) (Fig. 4H). The number of neurites did not significantly change with secretion application or TNFα neutralization (Fig. 4I). Although severity of fibre disorganization was significantly increased with VS8 secretion application, it could not be prevented by TNFα neutralization (Fig. 4J). Our results suggest TNFα as a key player in causing hearing loss due to VS and that neutralization of TNFα should be explored as a preventive therapy against VS-induced SNHL.

Recombinant VEGF application leads to decreased HGF secretion without notable morphological changes in cochlear explants. Administering bevacizumab, a VEGF monoclonal antibody, to patients with NF2-associated VSs results in hearing improvement independent of tumour shrinkage[3,22]. The mechanism behind this improvement in hearing is currently unknown. To understand the role of VEGF in causing SNHL due to VS, we applied recombinant VEGF onto cochlear explants. No significant loss of IHCs (Fig. 5B), OHCs (Fig. 5C), neurites (Fig. 5D) or fibre organization (Fig. 5E) was noted in the apical (n = 5) and basal turns (n = 3) when compared to NT controls (n = 4). However, there was a trend for a decreased number of OHCs in the apical and basal turns, and a trend for a decreased number of neurites in the apical turn. Representative projection images of control apical and basal turns and VEGF-A-treated apical and basal turns are shown in Fig. 5A (a–d respectively). Osmolality did not deviate from control media, being 330 mOsm/kg for both control media and media with recombinant VEGF-A.

Previous work from our laboratory demonstrating cross-talk between VEGF and hepatocyte growth factor (HGF) in primary human vestibular schwannoma and Schwann cells[23] motivated us to explore cross-talk between VEGF-A and HGF in cochlear explants as a potential mechanism through which VEGF-A can cause SNHL due to VS. HGF
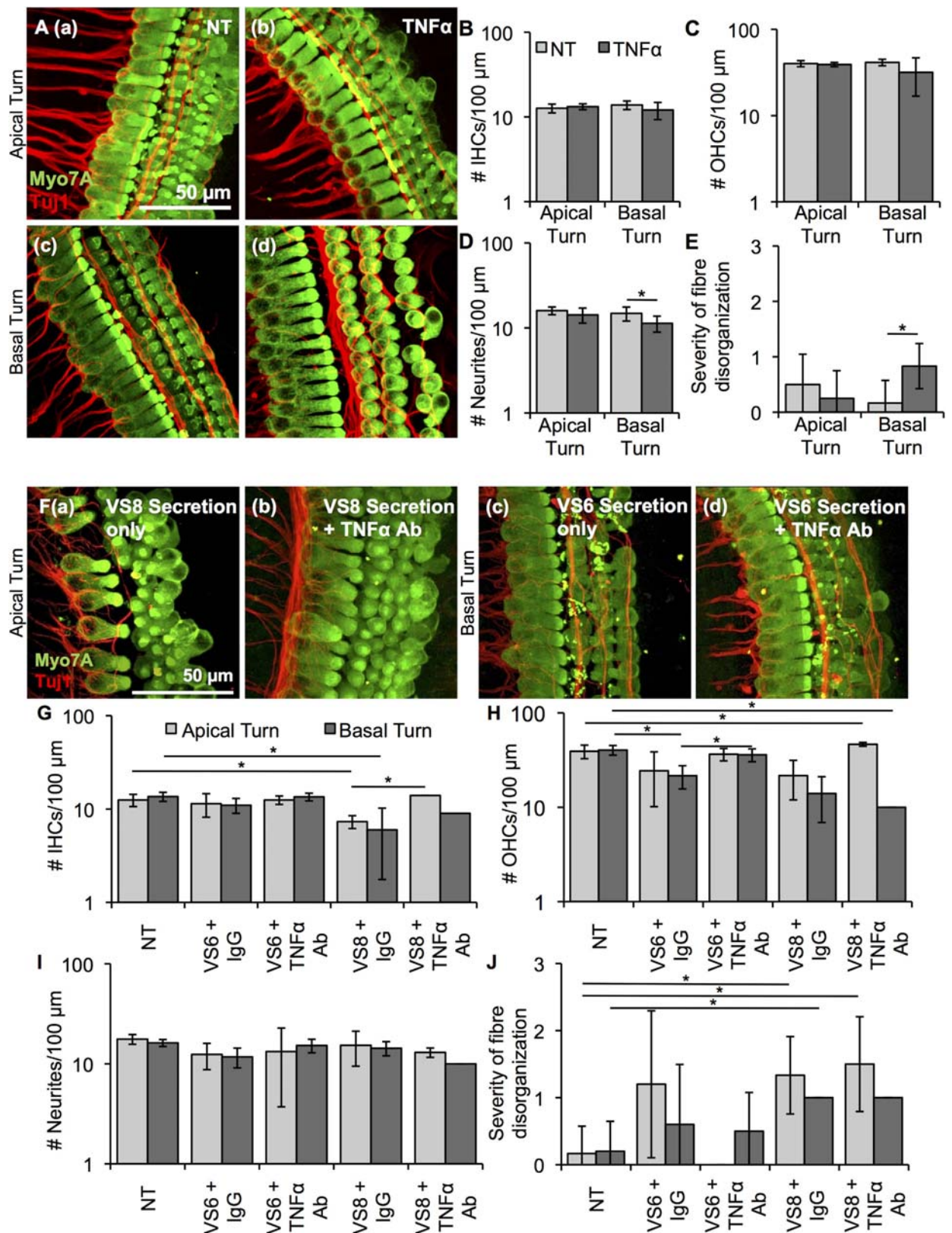
Figure 4. TNFα application onto cochlear explants induced mild damage. (A). Representative images for cochlear explants receiving no treatment (NT, a, c, n = 6 different explants) or incubated with TNFα (b, d, n = 4–6 different explants) are shown for the apical (a, b) and basal (c, d) turn. Myo7A (green) marks hair cells and Tuj1 (red) marks neurites. Scale Bar = 50 μm applies to all images. **(B)**. Number of inner hair cells (IHCs), **(C)**. number of outer hair cells (OHCs), **(D)**. number of neurites, **(E)**. severity of fibre disorganization are shown for a 100 μm length within the apical and basal turn explants for NT (light grey columns) and TNFα-treated (dark grey columns). *$p < 0.05$. **(F)**. Representative images for cochlear explants receiving VS secretions only (a, c, n = 2 different tumours) or incubated with VS secretions with TNFα antibody (b, d, n = 2 different

tumours) are shown for the apical (a, b) and basal (c, d) turn. Myo7A (green) marks hair cells and Tuj1 (red) marks neurites. Scale Bar = 50 μm applies to all images. (**G**). Number of inner hair cells (IHCs), (**H**). number of outer hair cells (OHCs), (**I**). number of neurites, (**J**). severity of fibre disorganization are shown for a 100 μm length within the apical and basal turn explants treated with VS secretions only (light grey columns) and VS secretions with TNFα antibody (dark grey columns). *p < 0.05.

is an interesting molecule because its levels in the cochlea are tightly regulated: too much or too little HGF causes SNHL[24]. To understand VEGF-A and HGF's relationship in cochlear cells, HGF levels were measured after VEGF-A addition. Basal secreted HGF levels were 48.1 ± 17.0 pg/mL in control cochlear explants (n = 4, Fig. 5F). Treating cochlear explants with 5 μg/mL recombinant human VEGF-A for 48 hours led to significantly lower levels of HGF, being 10.5 ± 9.4 pg/mL (n = 4, p = 0.01, Fig. 5F). This change was specific to VEGF-A since incubating the cochlear explants with the same concentration of TNFα did not lead to changes in the secreted HGF levels (n = 3, p = 0.40).

Discussion

We show, for the first time, that VS-secreted factors can lead to cochlear degeneration. Our study was motivated by previous work that demonstrates the lack of correlation between the radiographic tumour size or tumour extent within the internal auditory canal and audiometric threshold shifts in people with sporadic VS[5,6]. Our results support our hypothesis that secreted factors from VS can lead to oto- and neurotoxicity. While application of solely culture medium or secretions from healthy GANs led to no detectable cochlear damage (with the exception of mild fibre disorganization noted in response to secretions from only 1 control nerve), secretions from different tumours resulted in variable patterns of cochlear damage *in vitro*, providing mechanistic insight into the variable degree of cochlear histopathology observed *post mortem* in people with VS[5]. Overall, the damage noted in the cochlear explants after VS secretion application was reflective of the patient's degree of ipsilateral SNHL. For instance, VS8, from a patient with a deaf ear, led to drastic degeneration of the cochlea, including loss of HCs and neurites, changes that would cause profound SNHL, with increasing severity from the apical to basal turn as commonly noted in VS patients[1].

In contrast to VS secretions from a patient with ipsilateral anacusis, many other VS secretions caused more specific damage: secretions from VS6, removed from a subject with a moderate SNHL, demonstrated specific loss of OHCs in the basal turn, potentially explaining the elevated pure tone average of 40 dB, as OHC dysfunction is thought to lead to 40–60 dB threshold shift in hearing[25]. The patient's mild decrease in word recognition on the ipsilateral side suggests potential dysfunction of the neural pathway along with HC loss, a pathology that could be explained through neurite loss noted in the cochlear explant cultures.

Interestingly, although VS7 and VS8 patients presented with an essentially deaf ear, their secretions led to very different changes in the cochlear explants, with VS7 secretions causing primarily fibre disorganization. There could be several reasons for this difference. First, VS7 secretions' induction of substantial fibre disorganization could have led to the audiometric results noted (≥100 dB pure tone average and 0% word recognition) even with the rest of the cochlear structures being intact. If the fibres are disorganized and dysfunctional, then the information is not transmitted from the HCs to the more central auditory centres. Indeed, synaptic loss and disorganization, without loss of HCs or neurons, has been described in other pathologies characterized by SNHL, including Meniere's disease[26]. Clinically, if DPOAE testing, which reflects outer hair cell function, was available in the patient with VS7, we would have expected it to be normal despite profound deafness, as observed in people with auditory neuropathy[27]. Second, as the patient with VS7 also had a history of exposure to bilateral loud noise, and moderate SNHL contralaterally (34 dB pure tone average, 84% word recognition), the ipsilateral ear could have been more vulnerable to VS than if the ear had not been acoustically traumatized. Third, as we hypothesize that the SNHL due to VS is multifactorial, different VSs could cause SNHL in different ways[1]. The main mechanism behind the SNHL due to VS7 could be more so nerve compression or tumour-associated oedema rather than secreted factors, whereas for VS8, it could be mainly due to the secreted factors. However, auditory nerve compression by VS7 is unlikely to be the main mechanism of SNHL because VS7 was substantially smaller than VS8 *in vivo*, and the underlying assumption is that extent of compression is correlated with tumour size. Other than the tumour's physical size, there could be other factors influencing VS-mediated SNHL. In our study, three of the younger subjects had the best pure-tone hearing, albeit another young patient suffered from anacusis (tumour VS10). Subjects with good hearing were significantly younger than patients with poor hearing (Fig. 1). Age and gender effects on VS secretions have not been studied in the past. Nonetheless, age of patients has been found to correlate with level of hearing loss[28]. In another study, females with VS seem to have a lower incidence of hearing loss (HL) and smaller tumours than males[29]. It could be possible that age or gender of the subject as well as age of the tumour are influencing the profile of secreted proteins from these tumours. Future work is warranted to investigate the age and gender effects in order to gain a better understanding of the factors influencing these tumours' ototoxic and neurotoxic ability.

To delineate these various possibilities, our findings strongly motivate future clinical studies that would entail serial audiometric testing, including DPOAE testing, combined with serial collection of perilymph, CSF and serum in VS patients.

In the meantime, our model and hypotheses are strengthened by the results that secretions from all three VS associated with GH or GANs did not lead to significant HC or neurite loss in the cochlear explants. Interestingly, VS2 and VS5, tumours associated with GH, did cause significant fibre disorganization in the only apical turn or basal turn, respectively. It may be that the putative damage due to VS5 secretions *in vivo* was in the basal region encoding frequencies higher than the highest frequency tested by threshold audiometry. Early, asymptomatic SNHL can be detected when hearing is tested above the frequency range evaluated regularly (≤8 kHz)[30]. It may be possible that secretions from healthy nerves or GH tumours do not lead to significant fibre disorganization *in vivo*
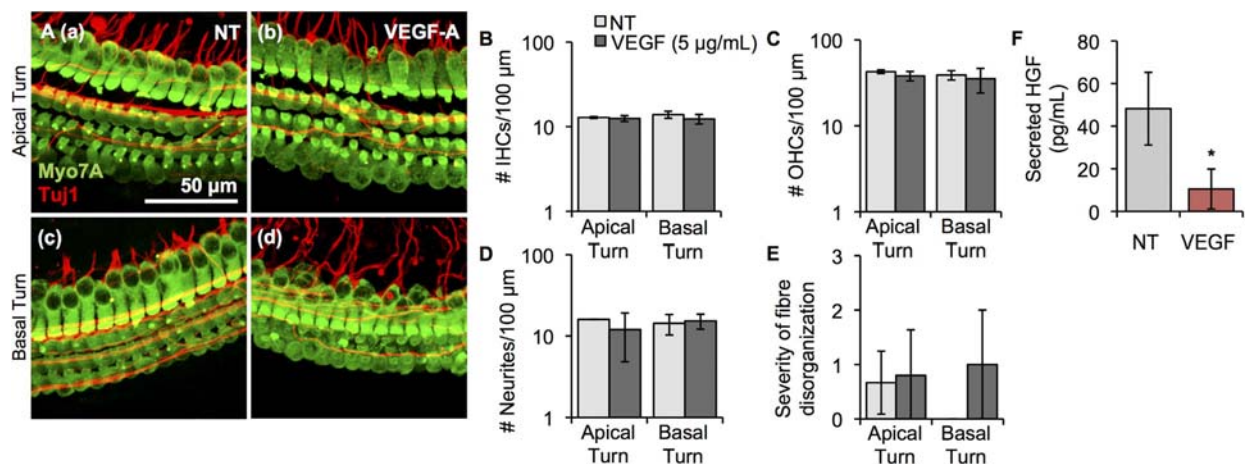
Figure 5. VEGF-A application onto cochlear explants did not induce substantial cellular damage but did significantly decrease HGF secretion. (**A**). Representative images for cochlear explants receiving no treatment (NT, a, c, n = 3–4 different explants) or incubated with VEGF-A (b, d, n = 3–5 different explants) are shown for the apical (a, b) and basal (c, d) turn. Myo7A (green) marks hair cells and Tuj1 (red) marks neurites. Scale Bar = 50 μm applies to all images. (**B**). Number of inner hair cells (IHCs), (**C**). number of outer hair cells (OHCs), (**D**). number of neurites, (**E**). severity of fibre disorganization are shown for a 100 μm length within the apical and basal turn explants for NT (light grey columns) and VEGF-A-treated (dark grey columns) (**F**). Secreted murine HGF levels in cochlear explants post-VEGF treatment. *p < 0.05.

due to counteracting homeostatic mechanisms that maintain fibre organization, a feature that was lacking in our *in vitro* model. Nonetheless, the lack of cochlear cell loss due to application of secretions from VS of subjects with GH or GAN secretions provides confidence that the degeneration seen is due to the specific molecules present in the VS secretions, rather than due to the paradigm.

Using a candidate molecule approach, we focused on two proteins that may be orchestrating SNHL due to VS: TNFα as a putative ototoxic molecule, and FGF2 as a putative otoprotective molecule. We found, for the first time, the robust correlation between TNFα levels in VS secretions and degree of SNHL, in terms of both pure tone average and word recognition. This result is intriguing because TNFα is known to cause SNHL in other aetiologies, based on elevated TNFα serum levels in people with idiopathic sudden SNHL[31] and immune-mediated SNHL[32]. Similar to VS secretions causing more severe damage in the basal than apical turn, TNFα-induced damage in IHCs and nerve fibres specifically in the basal turn. Applying TNFα onto the cochlear explants did not induce severe damage as has been described previously when applying TNFα at the same concentration (1 μg/mL) to rat cochlear explants[33]. This is most likely because we did not use the lowest most part of the basal turn near the hook region, where Dinh *et al.* noted most of the damage[33]. We also cultured the cochlear explants intact with neuronal connections that could provide growth factors and protective molecules, whereas Dinh *et al.* cultured only the organ of Corti. Differences could also be due to variability in species susceptibility (mouse versus rat), because we used TNFα from a different species (human) than the derived cochlear explants (mouse), or because of differences in source and purity of recombinant TNFα compared to previous studies. Nonetheless, our study is the first to identify an ototoxic secreted molecule in the context of VS. Probing TNFα's precise role in VS-mediated SNHL, we assessed whether antibody-mediated TNFα neutralization could prevent cochlear explant damage noted with secretion application. Intriguingly, TNFα neutralization could prevent IHC loss due to one tumour's secretions (VS8) and prevent OHC loss (VS6) in another case. It is possible that TNFα has differing ototoxic effects based on a given tumour's secretome or other properties of the tumour. TNFα inhibition via monoclonal antibodies has been clinically successful in alleviating immune-mediated and idiopathic SNHL[34], and our study suggests that it could also be beneficial for VS-mediated SNHL.

In contrast to the positive correlation between TNFα levels in VS secretions and degree of SNHL, we found a negative correlation between FGF2 levels in VS secretions and degree of SNHL. This is interesting because FGF2 is a known otoprotective molecule in other contexts[35,36]. Although we previously reported on this correlation for FGF2, when analyzing the *average* levels of FGF2 in VS secretions associated with good versus PH[21], we now provide additional insight into the strength of this correlation by plotting *individual* FGF2 levels from VS secretions and comparing them with pure tone average and word recognition from the same subject. Taken together, our data suggest that subjects whose VS secrete high levels of FGF2 and low levels of TNFα will most likely retain their hearing even while having the VS. Our results motivate future development of a prognostic test for SNHL due to VS, possibly based on serum or CSF levels of TNFα and FGF2.

Based on the therapeutic efficacy and unknown mechanism of anti-VEGF-A antibody, bevacizumab, in rescuing SNHL in some NF2 patients with VS[3], we explored whether exogenous VEGF-A application to cochlear explants could be toxic. We found no morphologic damage of cochlear cells exposed to exogenous VEGF-A. Interestingly, we did find that VEGF-A application specifically decreased the explants' HGF secretion. This is significant because human mutations in HGF are associated with nonsyndromic SNHL[24], while mice that lack or overexpress HGF are deaf[24], indicating that cochlear HGF levels need to be carefully controlled to assure normal hearing. Increased

cochlear VEGF-A expression has been described in various cell types, including spiral ganglion neurons and the stria vascularis, after different inner ear insults[37], including after noise trauma[38] and vibration-induced SNHL in guinea pigs[39]. Importantly, application of recombinant HGF to cochlear explant cultures has been shown to significantly reduce the HC loss induced by aminoglycosides, and local application of HGF to the round window membrane of guinea pigs attenuates noise-induced HL[40]. Therefore, increasing HGF levels by VEGF-A inhibition could be otoprotective and potentially be the mechanism of hearing improvement with bevacizumab in NF2 VS patients. It also may be that VEGF-A is acting along with several other molecules, such as TNFα or FGF2, to create a cumulative ototoxicity profile due to VS. Further, limitations of our model system such as usage of immature cochlear explants and the ability of only short-term assessment could have produced divergent results *in vitro* versus what is noted *in vivo* although others have demonstrated the utility of the neonatal cochlear explant system for screening of drugs that also worked in the mature cochlea *in vivo*[41,42].

We demonstrate, for the first time, direct cochlear damage due to VS-secreted factors. Tumour secretions led to variable types of damage, including loss of hair cells and neurites, and neurite disorganization; the damage tended to increase in severity from apical to the basal turn, and correlated with the severity of SNHL. These results with cochlear explants provide mechanistic insight into the variable damage of cochlear cells noted in *post mortem* histopathologic analyses of human temporal bones with untreated VS, where cochlear damage was much more severe in VS associated with poor than with GH[10]. We identified VS-secreted TNFα and FGF2 as some of the first ototoxic and otoprotective molecules, respectively, which may be modulating SNHL due to VS. Our results support the hypothesis that VS-secreted factors can damage or protect cochlear cells, controlling the SNHL in VS patients.

Methods

Study population and human specimen collection.
Surgical VS specimens were collected from patients with sporadic VSs. Control great auricular nerves (GANs) were obtained from patients undergoing neck dissections or parotidectomies, during which these nerves are routinely sacrificed. Informed consent was obtained from all patients. The study protocols were approved by the Human Studies Committee of Massachusetts General Hospital and Massachusetts Eye and Ear Infirmary, and conducted in accordance with the Helsinki Declaration. We utilized GAN as the control for the following reasons: (1) GAN is a sensory nerve like the vestibular nerves with a robust sheath of Schwann cells; (2) tumours developing on this nerve are exceptionally rare (we could not find any schwannomas on the GAN described in the literature), making it unlikely that the tissue we utilized had neoplastic properties; (3) healthy GANs are routinely sacrificed during neck surgery for access to deeper neck structures so we have ready access to control tissue, (4) the cochlear nerve that is sometimes sacrificed during resection of the adjacent VS cannot be assumed to be "healthy" because it shares the tumour microenvironment with the VS, and it may harbor pre-tumourous molecular changes as it is anatomically contiguous with the vestibular nerves at the root entry zone of CNVIII. Specimens were placed in sterile saline on ice for 15 minutes while being transported to the laboratory. Age was defined at the time of diagnosis. Tumour size (largest diameter parallel to the petrous face), PTA (the average of the lowest thresholds (in dB) for two tones among 0.5, 1 and 2 kHz) and WR (the percentage of spoken words a subject can comprehend) were from the last measurements prior to tumour surgery. A deaf ear was assigned a PTA of 100 dB and WR of 0%.

Preparation of vestibular schwannoma secretions.
After separating cauterized and hemorrhagic parts of the fresh sporadic VS or GAN specimens, the sample was rinsed with sterile PBS thrice. VS and GAN secretions were collected by incubating a washed, fresh tumour or control specimen in 100% DMEM for 3 days at 37°C and 5% CO_2 levels in sterile conditions. The secretions were normalized by weight (0.1 g specimen/0.1 mL DMEM). In addition, DMEM alone was incubated in parallel as control media (used for no treatment (NT)). Following the removal of the tumour piece, secretions were frozen at −80°C until further use.

Murine cochlear explant culture.
To develop an animal model to study SNHL due to VS, cochlear explant cultures were established from CBA/CaJ mice (Jackson Laboratory, ME) on postnatal day 3–5. The study protocol was approved by the Institutional Animal Care and Use Committee of Massachusetts Eye and Ear Infirmary. Briefly, after decapitation, temporal bones were surgically separated from the skull in Hank's Balanced Salt Solution (Life Technologies, NY). The bony otic capsule encasing the cochlea was dissected away. The spiral ligament was carefully stripped away along the cochlear length, starting from the base, to expose the organ of Corti with sensory hair cells connected to the spiral ganglion neuron region. The lower apical and upper basal turns were microdissected separately, providing two pieces per cochlea. The upper apical turn and hook of the basal turn were discarded as they were often damaged during dissection. For brevity, the lower apical and upper basal turns are referred to as "apical" and "basal" in the remainder of manuscript. Reissner's membrane was removed. The explants were cultured onto 10 mm glass coverslips coated with Cell-Tak (BD Biosciences, CA, #354241) in a 35 mm culture dish with 4 wells. Culture medium consisted of 98% DMEM, 1% ampicillin and 1% N2 supplement.

Treatment of cochlear explant cultures with vestibular schwannoma or healthy nerve secretions.
After approximately 12 hours of culturing, the cochlear explant cultures were treated for 48 hours with tumour or control healthy nerve (without schwannomas) secretions diluted to half their concentration. The exact formulation was 46.5% tumour- or nerve-conditioned DMEM, 46.5% plain DMEM, 5% FBS, 1% ampicillin and 1% N2 supplement. All reagents were purchased from Life Technologies, NY. Importantly, for any experimental treatment, control explants receiving no treatment (NT, composed of 46.5% DMEM incubated without specimen for 3 days simultaneously as DMEM incubated with specimen, 46.5% plain DMEM, 5% FBS, 1% ampicillin and 1% N2 supplement)) from age-matched littermates were analyzed in parallel.

In order to understand TNFα's effect on cochlear damage due to VS secretions, TNFα neutralization was conducted in two tumour secretions (VS6 and VS8) by incubating secretions in a goat anti-TNFα antibody (R&D Systems) for 2 h prior to application onto cochlear explants. TNFα neutralization was assessed in VS6 secretions after incubation with anti-TNFα antibody through ELISA (R&D Systems). Control experiments encompassed incubating NT media with anti-TNFα antibody, or incubating VS secretions or NT media with goat IgGs using the same protocol. After incubation with TNFα antibodies or goat IgGs, VS secretions were applied along with the antibody to cochlear explants for 48 h before fixation for immunofluorescence and imaging.

Measurements of secretions.   Osmolality in VS secretions was measured using a vapor-pressure osmometer (standardized with 290 mOsm/kg of 0.1567M sodium chloride solution) before applying them onto cochlear explants.

Extra formulation for some VS secretions (collected either in PBS for 1 hour or in DMEM for 3 days) was made to be tested on ELISA. Human TNFα and FGF2 ELISAs (R&D Systems) were conducted as directed by the manufacturer on VS secretions prior to being applied onto the cochlear explant cultures. Mouse HGF ELISA kit was purchased from R&D systems and manufacturer's protocols were followed.

Treatment of cochlear explant cultures with recombinant TNFα and VEGF.   Recombinant human TNFα or human VEGF (R&D Systems), diluted in culture media to a 5 μg/mL concentration, was applied to cochlear explants for 48 hours. Control cochlear explants received the same volume of PBS in the media as needed to add recombinant TNFα. The culture media was saved for analysis after treatment and the explants were fixed for immunofluorescence.

Immunofluorescence and imaging.   After treatment, cochlear explants were rinsed in phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde (Electron Microscopy Sciences, PA) in PBS for 20 minutes, washed with PBS, treated for 5 minutes, exposed to a blocking buffer consisting of 5% Normal Horse Serum (NHS, Sigma-Aldrich, MO) with 1% Triton-X (Integra Chemical, WA) for 1 hour, and incubated with antibodies against myosin 7A (Myo7A, Proteus Biosciences, CA, 1:200) and β-tubulin (Tuj1, Covance, MA, 1:200) diluted in 1% NHS with 1% Triton-X overnight at 4 °C, to identify hair cells (HCs) and neurons, respectively. After PBS washes, secondary antibodies (Alexa Fluor 555 anti-mouse and Alexa Fluor 488 anti-rabbit, Life Technologies, 1:1000) diluted in 1% NHS with 1% Triton-X were applied for 2 hours. Nuclear staining was performed with one 2-minute immersion in Hoechst stain 33342 (Life Technologies, NY, 1 nM dilution) followed by two 5-minute PBS washes. The coverslips were mounted on glass slides using Vectashield (Vector Laboratories, CA, #H-1000). The edges of the coverslips were sealed with clear nail polish (Electron Microscopy Sciences, PA). Cochlear explants were imaged using a Leica TCS SP5 Confocal microscope. Zoomed-in pictures for the region of organ of Corti, including neurites, were merged in a z-stack to obtain a z-axis projection image in the Leica software. The number of inner (IHCs) and outer hair cells (OHCs) and neurites were manually counted per 100 μm length along the cochlea, with 1–2 samplings per specimen. The 100 μm section for analysis was used for all analyses as it was deemed a long enough length for an accurate count but a short enough length for it to be consistently evaluated between specimens and has been commonly used in previous literature (e.g.[41]). The neurites were counted and assessed approximately 10 μm beneath the inner hair cells. Fibre organization, including both afferent radial neurites from the IHCs and efferent spiralling neurites to the OHCs, was assessed using a qualitative scale with 0 being essentially intact and 2 equalling severe disorganization. Supplementary Figure 2 illustrates the differences between a specimen assigned severities of 0,1 and 2 and highlights two regions within the image where neurite disorganization was assessed, i.e. the afferent radial neurites and the efferent spiraling neurites. Disorganization was defined as being 0 if the majority of the neurites were aligned perpendicularly to the hair cells' horizontal axis, were easily distinguishable from each other and were not very tortuous. Disorganization was defined as being 1 if the neurites were not aligned perpendicularly to the hair cells' horizontal axis, neurites were tangled with other neighboring neurites and were more tortuous. Disorganization was defined as being 2 if the neurites had a beading or tortuous appearance (i.e. the path of the neurites to the hair cells was not apparent), were not aligned with each other or perpendicularly aligned to the hair cells, and the ends of the neurites generally did not terminate at the basal parts of the hair cells. All assessments and analyses were conducted in a blinded-fashion.

Statistical analyses.   For the cytokine array, proteins were determined to be significantly expressed if the corresponding spots had optical densities more than 2 standard deviations of background values above the mean background level for each array. For the cytokine array and ELISA, statistical significance was determined through an analysis of variance (ANOVA) with alpha set to 0.05. For all cochlear explant experiments, differences were analyzed using a two-tailed t-test. For application of secretions, Benjamini-Hochberg correction for multiple hypotheses was conducted on generated p-values and Benjamini-Hochberg corrected p-values ≤ 0.05 were considered significant.

References

1. Thakur, J. D. *et al.* An update on unilateral sporadic small vestibular schwannoma. *Neurosurg. Focus* **33,** E1, doi: 10.3171/2012.6.FOCUS12144 (2012).
2. Mahaley, M. S., Jr., Mettlin, C., Natarajan, N., Laws, E. R., Jr. & Peace, B. B. Analysis of patterns of care of brain tumor patients in the United States: a study of the Brain Tumor Section of the AANS and the CNS and the Commission on Cancer of the ACS. *Clin. Neurosurg.* **36,** 347–352 (1990).
3. Plotkin, S. R. *et al.* Bevacizumab for progressive vestibular schwannoma in neurofibromatosis type 2: a retrospective review of 31 patients. *Otol. Neurotol.* **33,** 1046–1052, doi: 10.1097/MAO.0b013e31825e73f5 (2012).

4. Aronzon, A., Ruckenstein, M. J. & Bigelow, D. C. The efficacy of corticosteroids in restoring hearing in patients undergoing conservative management of acoustic neuromas. *Otol. Neurotol.* **24,** 465–468 (2003).
5. Nadol, J. B., Jr., Diamond, P. F. & Thornton, A. R. Correlation of hearing loss and radiologic dimensions of vestibular schwannomas (acoustic Neuromas). *Am. J. Otol.* **17,** 312–316 (1996).
6. Caye-Thomasen, P., Dethloff, T., Hansen, S., Stangerup, S. E. & Thomsen, J. Hearing in patients with intracanalicular vestibular schwannomas. *Audiol. Neurootol.* **12,** 1–12, doi: 10.1159/000096152 (2007).
7. Mackay, I. S. & King, I. J. Pre- and post-operative brainstem responses in a case of acoustic neuroma, sparing the VIII nerve. *Clin. Otolaryngol. Allied Sci.* **2,** 233–238 (1977).
8. Thomsen, J., Terkildsen, K. & Osterhammel, P. Auditory brain stem responses in patients with acoustic neuromas. *Scand. Audiol.* **7,** 179–183 (1978).
9. Gouveris, H. T., Victor, A. & Mann, W. J. Cochlear origin of early hearing loss in vestibular schwannoma. *Laryngoscope* **117,** 680–683, doi: 10.1097/MLG.0b013e31803146c5 (2007).
10. Roosli, C., Linthicum, F. H., Jr., Cureoglu, S. & Merchant, S. N. Dysfunction of the cochlea contributing to hearing loss in acoustic neuromas: an underappreciated entity. *Otol. Neurotol.* **33,** 473–480, doi: 10.1097/MAO.0b013e318248ee02 (2012).
11. Stankovic, K. M. *et al.* Genetic determinants of hearing loss associated with vestibular schwannomas. *Otol. Neurotol.* **30,** 661–667, doi: 10.1097/MAO.0b013e3181a66ece (2009).
12. Lassaletta, L., Martinez-Glez, V., Torres-Martin, M., Rey, J. A. & Gavilan, J. cDNA microarray expression profile in vestibular schwannoma: correlation with clinical and radiological features. *Cancer Genet. Cytogenet.* **194,** 125–127, doi: 10.1016/j.cancergencyto.2009.06.016 (2009).
13. Silverstein, H. A rapid protein test for acoustic neurinoma. *Arch. Otolaryngol.* **95,** 202–204 (1972).
14. Silverstein, H. Labyrinthine tap as a diagnostic test for acoustic neurinoma. *Otolaryngol. Clin. North Am.* **6,** 229–244 (1973).
15. van de Langenberg, R. *et al.* Predictors of volumetric growth and auditory deterioration in vestibular schwannomas followed in a wait and scan policy. *Otol. Neurotol.* **32,** 338–344, doi: 10.1097/MAO.0b013e3182040d9f (2011).
16. Asthagiri, A. R. *et al.* Mechanisms of hearing loss in neurofibromatosis type 2. *PloS One* **7,** e46132, doi: 10.1371/journal.pone.0046132 (2012).
17. Lysaght, A. C. *et al.* Proteome of human perilymph. *J. Proteome Res.* **10,** 3845–3851, doi: 10.1021/pr200346q (2011).
18. Yuan, Y. *et al.* Ouabain-induced cochlear nerve degeneration: synaptic loss and plasticity in a mouse model of auditory neuropathy. *J. Assoc. Res. Otolaryngol.* **15,** 31–43, doi: 10.1007/s10162-013-0419-7 (2014).
19. Juhn, S. K., Prado, S. & Rybak, L. Effect of urea on osmolality of perilymph. *Arch. Otolaryngol.* **105,** 538–541 (1979).
20. Oishi, N. *et al.* Tumor necrosis factor-alpha-mutant mice exhibit high frequency hearing loss. *J. Assoc. Res. Otolaryngol.* **14,** 801–811, doi: 10.1007/s10162-013-0410-3 (2013).
21. Dilwali, S. *et al.* Sporadic vestibular schwannomas associated with good hearing secrete higher levels of fibroblast growth factor 2 than those associated with poor hearing irrespective of tumor size. *Otol. Neurotol.* **34,** 748–754, doi: 10.1097/MAO.0b013e31828048ec (2013).
22. Plotkin, S. R. *et al.* Hearing improvement after bevacizumab in patients with neurofibromatosis type 2. *N. Engl. J. Med.* **361,** 358–367, doi: 10.1056/NEJMoa0902579 (2009).
23. Dilwali, S., Roberts, D. & Stankovic, K. M. Interplay between VEGF-A and cMET signaling in human vestibular schwannomas and schwann cells. *Cancer Biol. Ther.* **16,** 170–175, doi: 10.4161/15384047.2014.972765 (2015).
24. Schultz, J. M. *et al.* Noncoding mutations of HGF are associated with nonsyndromic hearing loss, DFNB39. *Am. J. Hum. Genet.* **85,** 25–39, doi: 10.1016/j.ajhg.2009.06.003 (2009).
25. Liberman, M. C. *et al.* Prestin is required for electromotility of the outer hair cell and for the cochlear amplifier. *Nature* **419,** 300–304, doi: 10.1038/nature01059 (2002).
26. Nadol, J. B., Jr. Innervation densities of inner and outer hair cells of the human organ of Corti. Evidence for auditory neural degeneration in a case of Usher's syndrome. *ORL J. Otorhinolaryngol. Relat. Spec.* **50,** 363–370 (1988).
27. Starr, A., Picton, T. W., Sininger, Y., Hood, L. J. & Berlin, C. I. Auditory neuropathy. *Brain.* **119** (Pt 3), 741–753 (1996).
28. Matthies, C. & Samii, M. Management of 1000 vestibular schwannomas (acoustic neuromas): clinical presentation. *Neurosurgery* **40,** 1–9; discussion 9–10 (1997).
29. Harun, A., Agrawal, Y., Tan, M., Niparko, J. K. & Francis, H. W. Sex and age associations with vestibular schwannoma size and presenting symptoms. *Otol. Neurotol.* **33,** 1604–1610, doi: 10.1097/MAO.0b013e31826dba9e (2012).
30. Lee, J. *et al.* Behavioral hearing thresholds between 0.125 and 2 kHz using depth-compensated ear simulator calibration. *Ear Hear.* **33,** 315–329, doi: 10.1097/AUD.0b013e31823d7917 (2012).
31. Demirhan, E. *et al.* Blood levels of TNF-alpha, IL-10, and IL-12 in idiopathic sudden sensorineural hearing loss. *Laryngoscope* **123,** 1778–1781, doi: 10.1002/lary.23907 (2013).
32. Svrakic, M. *et al.* Diagnostic and prognostic utility of measuring tumor necrosis factor in the peripheral circulation of patients with immune-mediated sensorineural hearing loss. *Arch. Otolaryngol. Head Neck Surg.* **138,** 1052–1058, doi: 10.1001/2013.jamaoto.76 (2012).
33. Dinh, C. T. *et al.* Dexamethasone protects organ of corti explants against tumor necrosis factor-alpha-induced loss of auditory hair cells and alters the expression levels of apoptosis-related genes. *Neuroscience* **157,** 405–413, doi: 10.1016/j.neuroscience.2008.09.012 (2008).
34. Lobo, D., Garcia-Berrocal, J. R., Trinidad, A., Verdaguer, J. M. & Ramirez-Camacho, R. Review of the biologic agents used for immune-mediated inner ear disease. *Acta Otorrinolaringol. Esp.* **64,** 223–229, doi: 10.1016/j.otorri.2012.04.008 (2013).
35. Zhai, S. Q. *et al.* Protective effect of basic fibroblast growth factor on auditory hair cells after noise exposure. *Acta Otolaryngol.* **122,** 370–373 (2002).
36. Zhai, S. Q., Wang, D. J., Wang, J. L., Han, D. Y. & Yang, W. Y. Basic fibroblast growth factor protects auditory neurons and hair cells from glutamate neurotoxicity and noise exposure. *Acta Otolaryngol.* **124,** 124–129 (2004).
37. London, N. R. & Gurgel, R. K. The role of vascular endothelial growth factor and vascular stability in diseases of the ear. *Laryngoscope* **124,** E340–346, doi: 10.1002/lary.24564 (2014).
38. Picciotti, P. M. *et al.* Vascular endothelial growth factor (VEGF) expression in noise-induced hearing loss. *Hear. Res.* **214,** 76–83, doi: 10.1016/j.heares.2006.02.004 (2006).
39. Zou, J., Pyykko, I., Sutinen, P. & Toppila, E. Vibration induced hearing loss in guinea pig cochlea: expression of TNF-alpha and VEGF. *Hear. Res.* **202,** 13–20, doi: 10.1016/j.heares.2004.10.008 (2005).
40. Inaoka, T. *et al.* Local application of hepatocyte growth factor using gelatin hydrogels attenuates noise-induced hearing loss in guinea pigs. *Acta Otolaryngol.* **129,** 453–457, doi: 10.1080/00016480902725197 (2009).
41. Mizutari, K. *et al.* Notch inhibition induces cochlear hair cell regeneration and recovery of hearing after acoustic trauma. *Neuron* **77,** 58–69, doi: 10.1016/j.neuron.2012.10.032 (2013).
42. Tong, M., Fernandez, K., Lall, K., Kujawa, S. & Edge, A. Increased Survival of Spiral Ganglion Neurons in Auditory Neuropathy by Treatment With Small Molecule Trk Receptor Agonists. *Association for Research in Otolaryngology MidWinter Meeting*, Abstract PS-570 (2014).

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Author Contributions

S.D. and K.M.S. designed research. S.D., L.D.L. and V.Y.R.S. performed experiments. S.D., L.D.L., V.Y.R.S. and K.M.S. analyzed data. D.G.D. provided surgical specimens. S.D. and K.M.S. wrote the manuscript. S.D., L.D.L., V.Y.R.S., D.G.D. and K.M.S. critically edited the manuscript.

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Neuro-Oncology

Extracellular vesicles derived from human vestibular schwannomas associated with poor hearing damage cochlear cells

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Background. Vestibular schwannoma (VS) is a tumor of the vestibular nerve that transmits balance information from the inner ear to the brain. Sensorineural hearing loss occurs in 95% of patients with these tumors, but the cause of this loss is not well understood. We posit a role of VS-secreted extracellular vesicles (EVs) as a major contributing factor in cochlear nerve damage.

Methods. Using differential centrifugation, we isolated EVs from VS cell line HEI-193 and primary cultured human VS cells from patients with good hearing or poor hearing. The EVs were characterized using a Nanosight device and transmission electron microscopy and by extracting their RNA content. The EVs' effects on cultured murine spiral ganglion cells and organotypic cochlear cultures were studied using a transwell dual-culture system and by direct labeling of EVs with PKH-67 dye. EV-induced changes in cochlear cells were quantified using confocal immunohistochemistry. Transfection of VS cells with a green fluorescent protein–containing plasmid was confirmed with reverse transcription PCR.

Results. Human VS cells, from patients with poor hearing, produced EVs that could damage both cultured murine cochlear sensory cells and neurons. In contrast, EVs derived from VS cells from patients with good hearing did not damage the cultured cochlear cells.

Conclusions. This is the first report on EVs derived from VSs and on the capacity of EVs from VSs from patients with hearing loss to selectively damage cochlear cells, thereby identifying a potential novel mechanism of VS-associated sensorineural hearing loss.

Keywords: cochlea, extracellular vesicles, sensorineural hearing loss, transwell culture, vestibular schwannoma.

Exosomes (including microvesicles and other vesicular structures released by cells, termed *extracellular vesicles* [EVs]) are cell-derived vesicles, measuring 30 to 200 nm, which contain the genetic profile of their cell of origin, including RNA, DNA, microRNA (miRNA), and proteins within a bilipid membrane.[1–3] EVs are produced by both normal and neoplastic cells[4] of virtually every organ origin and have been identified in virtually every biofluid.[5] EVs are thought to be important mediators of intercellular communication by transferring their cargo between cells, both locally and systemically.[6] EVs are emerging as important serum biomarkers of human diseases, including neurodegenerative diseases[7] and neoplasms[4] of the central nervous system, being able to accurately predict diseases 1–10 years before they become clinically manifest.[7] Moreover, EV-based therapeutics are being developed and have already entered the clinical arena.[8]

EVs produced by vestibular schwannomas (VSs) have not been previously reported. Yet VS, a tumor of the vestibular nerve that transmits balance information from the inner ear to the brain, is the fourth most common adult intracranial neoplasm.[9] VSs occur sporadically as unilateral tumors in 95% of patients or in association with hereditary neurofibromatosis

type 2 (NF2) syndrome, whose hallmark is bilateral VSs. VSs arise from myelin-producing Schwann cells of vestibular nerves due to somatic loss of function of merlin in NF2[10] and to other, as yet undefined, causes. Sensorineural hearing loss (SNHL) occurs in 95% of patients with these tumors,[11] but the cause of this hearing loss is not well understood. The hearing loss from VS growth results in part from tumor compression of the adjacent cochlear nerve that serves as a sensory conduction pathway. However, clinical observations suggest that there may be other explanations for the SNHL: (i) deafness in VS patients can occur suddenly, without change in tumor size[12]; (ii) large VSs may not cause hearing loss, while small ones may[13,14]; and (iii) the size of sporadic VSs within the internal auditory canal does not correlate with the extent of ipsilateral hearing loss[14,15] (albeit there is a correlation between VS size and hearing loss in NF2).[16,17] Histopathological examination of untreated VSs demonstrates ipsilateral cochlear damage, including loss of sensory inner hair cells (IHCs) (75% of specimens), outer hair cells (OHCs) (88%) and spiral ganglion neurons (SGNs) (85%).[13,14] This damage of cells in the cochlea, which is bathed by fluids in proximity to the VS, raises the hypothesis that tumor-induced deafness is related to tumor-secreted materials that bathe the cochlea. We have explored whether VS-derived EVs are mediators of this selective cochlear damage, as we have previously shown that genetic expression profiles of VSs associated with poor hearing (PH) differ from those of VSs associated with good hearing (GH).[18]

Materials and Methods

Human VS Cell Culture

A human NF2 VS-derived cell line immortalized with human papilloma virus E6-7 genes[19] was acquired from House Ear Institute (HEI-193). Primary VS cultures were derived from patients with sporadic VSs after obtaining informed consent. Experimental investigations of human and animal subjects were carried out according to the protocols approved by the Ethics and Human Studies Committee at the Massachusetts Eye and Ear and Massachusetts General Hospital (#196424-22, #717176, #15-003), while honoring the Declaration of Helsinki. Patients' ages were noted at the time of diagnosis. Two hearing metrics were used: (i) pure tone average, defined as the average of the lowest threshold (in dB) for 2 tones among 0.5, 1, and 2 kHz; and (ii) word recognition, defined as the percentage of spoken words from a standardized list a patient can comprehend in quiet. Primary VS cells were cultured as we previously described.[20]

Murine Spiral Ganglion Cell Culture

Spiral ganglion cell (SGC) culture was derived from CBA/CaJ mice purchased from the Jackson Laboratory. We used the standard culturing protocol in our laboratory,[21] with some modifications. Briefly, postnatal day P3-P5 cochleae were isolated and SGCs within the modiolus were dissected. The tissue was incubated in Hank's Balanced Salt Solution (Gibco), trypsin (2.5 mg/mL; Gibco), and collagenase IV (0.5 mg/mL; Sigma-Aldrich) at 37°C for 25 min. Enzymatic digestion was stopped by addition of 10% fetal bovine serum (FBS)

(Sigma-Aldrich). After centrifugation of suspended cells at 1000 g for 3 min at room temperature, the pellet was resuspended in culture medium, Dulbecco's modified Eagle's medium/F12 (Invitrogen) supplemented with 10% FBS, 1% ampicillin solution (Gibco), 1% GlutaMAX (Invitrogen), 5% sterile horse serum (NHS, Gibco), neurotrophin-3 16 ng/mL (Promega), brain-derived neurotrophic factor 8 ng/mL (Promega), and 2% B-27 supplement (Gibco). The cells were cultured for 24 h in 24-well culture plates (CytoOne) on glass slides treated with poly-L-lysine and laminin (BioCoat BD) before experimental treatment described below. A good dissection was confirmed with high purity of SGNs and Schwann cells as noted morphologically in the culture.

Murine Cochlear Explant Culture

Organotypic cochlear cultures (also known as cochlear explants) were prepared using a similar protocol as described for the culture of SGCs, with several modifications.[22,23] We focused on the middle part of cochlear turns because it consistently gave rise to robust, undamaged explants. Cochlear explants, consisting of the sensory epithelium with IHCs and OHCs, and the attached SGNs, were cultured on glass coverslips previously treated with Cell-Tak (BD Biosciences) to facilitate tissue adhesion. Cochlear explants were incubated at 37°C with 5% CO_2 for 24 h in Dulbecco's modified Eagle's medium, 1% ampicillin solution (Gibco), and 1% N2 supplement (Invitrogen), until experimental treatment. The dissections were initially confirmed by microscopically inspecting each specimen for the presence of morphologically neuron-like cells after culturing and by checking the organization and health of control cochlear explants receiving no treatment after the experiment was finished.

Cochlear explants were subjected to 3 different treatments: (i) 6 µL phosphate buffered saline (PBS) with EVs added to the culture medium, as described below; same volume of PBS served as a negative control; (ii) a dual-culture plate with 2 different concentrations of HEI-193 cells added to the top chamber (1×10^6 and 4×10^6 cells), with a membrane permeable to particles <1 µm in size (#353104, Fisher Scientific), which allowed cell-secreted microvesicles to cross the membrane; (iii) direct addition of EVs isolated from primary human VS tumors from patients with GH or PH. All cultures were incubated for 48 h.

Isolation of Extracellular Vesicles

Approximately 4×10^6 NF2 HEI-193 cells were cultured in 15-cm culture dishes as previously described.[20] After 24 h, the culture plate was washed with PBS. A culture medium was added as outlined above but supplemented with 5% EV-depleted FBS, purified by high-speed centrifugation to deplete EVs from the FBS. After 48 h, conditioned media were collected and centrifuged for 10 min at 300 g, then 10 min at 2000 g. The supernatant was filtered through a 0.8-µm filter (Millipore). EVs were pelleted by centrifugation at 100 000 g for 80 min in a Type 70 Ti Rotor (Beckman Coulter).

The primary human VS cells were cultured for a week. The culture medium was then replaced with 5% EV-depleted FBS for 48 h. The supernatant from each culture plate was collected

to purify and store EVs as described above. When EVs were applied to cochlear explants, the antibiotic penicillin/streptomycin was replaced with ampicillin in the culture medium, since ampicillin is not ototoxic.

Characterization of Extracellular Vesicles

The size distribution and concentration of EVs were assessed with the Nanosight LM20 machine (Malvern Instruments), while EVs' morphology and size were evaluated with transmission electron microscopy. The pellet rich in EVs was suspended in 50 μL PBS (filtered twice) at 4°C and fixed in 2% paraformaldehyde. A grid coated with carbon film was positioned on a suspension drop with EVs. The grids were placed directly on top of a drop of 2% uranyl acetate. The grids were examined with a Technai-12 transmission G2 Spirit Biotwin electron microscope (FEI).

RNA Extraction

For RNA extraction, 1×10^6 cells and EVs from 50 mL of media were used. Total RNA was extracted using the RNeasy Mini Kit (Qiagen), and small RNA was extracted using the miRNeasy Mini Kit (Qiagen) and the RNeasy MinElute Cleanup Kit (Qiagen), while adhering to the manufacturer's protocols. The quantitative and qualitative analysis of the extracted RNA was performed using the NanoDrop 2000 spectrophotometer (Thermo Scientific) and Agilent 2100 Bioanalyzer through an RNA 6000 Pico Kit (Agilent).

Reverse Transcription PCR

Reverse transcription (RT)-PCR was performed using SuperScript One-Step RT-PCR with Platinum Taq (Invitrogen), as detailed in the Supplementary materials.

Labeling of Extracellular Vesicles with PKH-67 dye

Plasma membrane and EVs derived from HEI-193 cells were labeled with PKH-67 dye (Sigma-Aldrich), following the manufacturer's protocol and as described by Ekstrom et al.[24] As the EVs have remnants of the cell membrane, the dye PKH-67 was present in secreted vesicles. EVs were suspended in 200 μL PBS after ultracentrifugation and labeled with PKH-67. To eliminate excess dye, the solution was washed 3 times using Vivaspin 2 tubes (Sartorius Stedim Biotech) for 3 min at 4000 g at room temperature. The solution was diluted with PBS and ultracentrifuged at 100 000 g for 90 min at 4°C. The pellet was diluted with 200 μL PBS; 20 μL of this new solution was added to the culture of SGCs. The number of cells internalizing labeled vesicles was quantified 4 h and 24 h later under light microscopy.

HEI-193 cells, referred to as NF2 cells, stained with PKH-67 were cultured in a dual-culture plate. SGCs, including neurons and Schwann cells, were cultured in the lower portion. The top chamber, with a membrane permeable to particles smaller than 1 μm, had 4×10^6 HEI-193 cells stained with PKH-67. After passing through the permeable membrane, the PKH-67–labeled EVs were internalized by cells in the lower chamber. The frequency of internalization of the labeled vesicles by SGCs was measured at 24 h and 48 h under light microscopy.

Transduction with pCT-CD63-GFP Plasmid

A plasmid containing green fluorescent protein (GFP) was used to evaluate whether the genetic material (RNA) present in EVs could be expressed by the receptor cells. HEI-193 cells were transfected with pCT-CD63-GFP plasmid (Cyto-Tracers, System Biosciences) using Lipofectamine 2000 Reagent (Invitrogen). The transduction efficiency was observed under an inverted microscope to identify living cells (Zeiss). Transfected cells were selected with puromycin 1 mg/mL (Santa Cruz Biotechnology). The EVs from these cells were isolated and RNA was extracted and reverse transcribed into cDNA by RT-PCR to assess the presence of GFP mRNA (168 bp amplicon).

Immunostaining of Cochlear Cells

Hair cells were labeled with the polyclonal rabbit primary antibody against myosin VIIA (myo7A) (Proteus Biosciences). SGNs and their nerve fibers were labeled with a monoclonal mouse primary antibody against class β-tubulin III (Tuj1) (BioLegend). Additional details are provided in the Supplementary materials. The Poly Caspase Assay Kit (Green, Neuromics) was used to stain apoptotic cells.

The specimens were imaged with a confocal microscope (Leica SP5), in 0.5-μm steps, according to a published protocol[25] with modifications. The samples were evaluated under magnification of 20×, 63×, and 126×. To capture representative morphology of each specimen, photographs were taken from the central region. In addition, various sectional slices were projected on a single plane using Leica software. The counting of IHCs, OHCs, and neural fibers was performed along 100 μm of cochlear length. The number and area of neuronal somata were quantified in a 1×10^4 μm² area using the ImageJ program. Specifically, for all cell types, an appropriately stained cell (eg, Myo7A stained hair cell) that was distinguishable as an individual cell with a discernible nucleus was counted regardless of cell size or degree of damage.

Statistical Analysis

We used Excel 2013 and GraphPad Prism 6 for Windows for statistical analyses. The D'Agostino–Pearson test was used to test normality of distribution. Student's t-test was applied as a parametric test. The Mann–Whitney and Kruskal–Wallis tests were applied as nonparametric tests. Dunn's multiple comparison test and Benjamini–Hochberg correction for multiple hypotheses were conducted on generated P-values. $P < .05$ was considered significant.

Results

Human Vestibular Schwannoma Cells Secrete Extracellular Vesicles

As EVs released by VS have not been previously reported, we first tried to isolate them from a human VS cell line. We used the HEI-193 cell line, which we refer to as NF2 cells, as this cell line was developed from a VS of a patient affected with NF2.[19] After differential centrifugation of cell-conditioned culture media, numerous small EVs were identified by transmission electron microscopy ($n = 2$; Fig. 1A). The size of the EVs
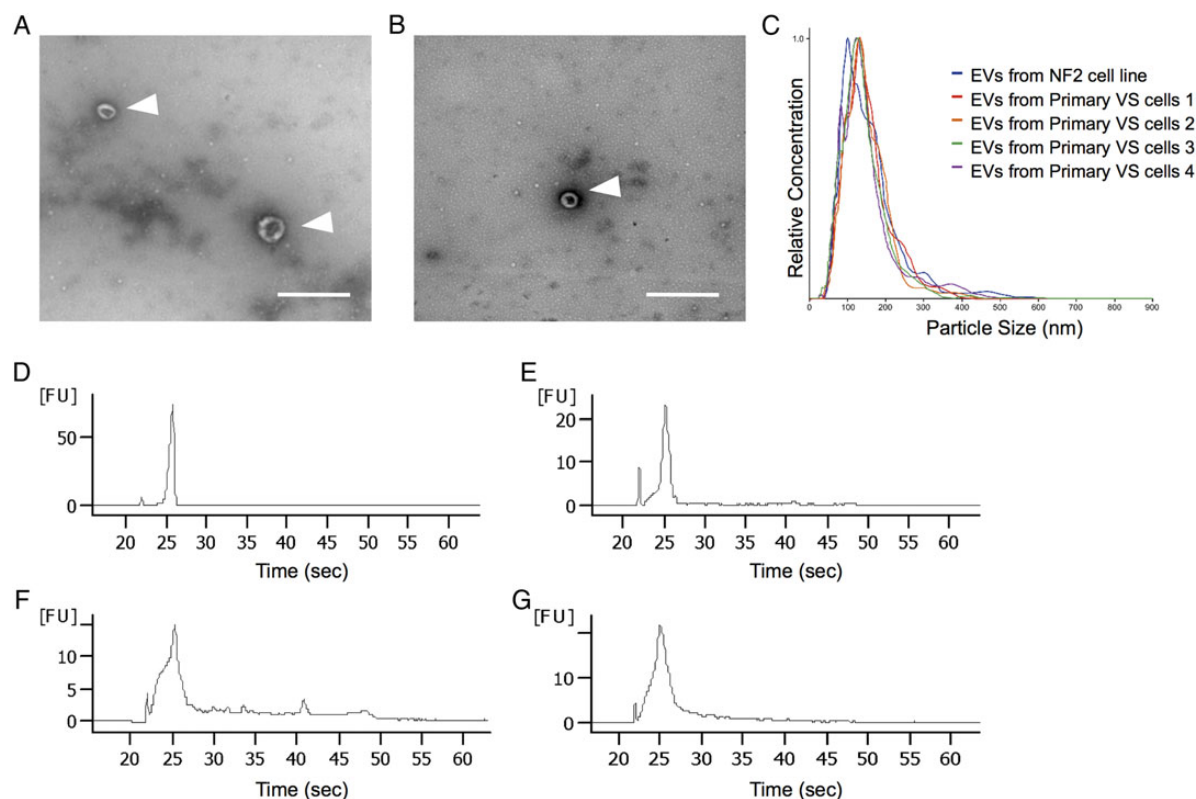
Fig. 1. VSs secrete EVs that contain small RNAs. Electron microscopy images show EVs in conditioned culture media from (A) the NF2 vestibular schwannoma cell line and (B) primary human VS cells. Round particles with defined edges resemble exosomes (marked with white arrowheads). (C) Particle distribution by size in conditioned culture media from the NF2 cell line and primary human VS cells ($n = 4$). The highest concentration is set to 1. Representative images of spectrophotometry of small RNA from cultured (D) HEI-193 cell line and (E) primary human VS cells. Representative images of spectrophotometry of small RNA present in isolated EVs from cultured (F) HEI-193 cell line and (G) primary human VS cells. FU, fluorescence unit. Scale bar: 500 nm (A, B).

ranged from 30 nm to 680 nm, and the most frequent size was 102 nm (Fig. 1C). Since we used EV-depleted media for our cultures, EVs detected were released from the HEI-193 cells. The distribution of particles in EV-depleted media is shown in Supplementary Fig. S1. The shape of the vesicles resembled upwardly facing cups of glass, consistent with previously described EVs.[26] Similarly shaped vesicles were observed in culture media in which primary human VS cells were grown ($n = 2$; Fig. 1B). These primary cells were obtained from indicated surgeries on patients with sporadic, unilateral VSs. The size distribution of EVs was similar between primary VS cells ($n = 4$) and NF2 cells (Fig. 1C).

Vestibular Schwannoma–Derived Extracellular Vesicles Contain RNA

It is known that RNA delivered through EVs can be functional—for example, mRNAs can be translated into proteins, and miRNAs can inhibit protein translation.[27] We found small RNAs in cultured NF2 cells ($n = 10$; Fig. 1D) and cultured primary VS cells ($n = 10$; Fig. 1E). The peak at 25s is consistent with miRNA, but could also reflect other small noncoding RNAs. We further found small RNA species in isolated EVs from NF2 cells ($n = 10$; Fig. 1F) and primary human VS cells ($n = 10$; Fig. 1G).

Spiral Ganglion Cells Can Internalize Tumor-Derived Extracellular Vesicles

To determine whether cochlear cells could internalize EVs secreted by VS cells (donor cells), PKH-67 dye that fluorescently labels cell membranes was used in 2 different experiments. First, EVs (5×10^{11} particles/μL) from 4×10^6 NF2 cells were stained with PKH-67 and added directly to the culture of dissociated murine SGCs (recipient cells) (Fig. 2A–D). The advantage of this monolayer of SGCs consisting of SGNs and Schwann cells is easy visualization of the internalized EVs by the recipient cells. Our focus on SGCs is motivated by the histopathological findings of a major loss of these cells in human temporal bones affected by VS.[13] Second, donor NF2 cells were stained with PKH-67 and placed in the top chamber of a dual-culture dish with cultures separated by a transmembrane filter and with a monolayer of murine SGCs in the bottom well (recipient cells) (Fig. 2E–G). As EVs form by budding of the bilipid cell membrane from the NF2 cells, they acquire PKH-67–stained membrane from their donor cells. These EVs cross the culture dish's permeable synthetic membrane, which allows particles sized <1 μm to pass through and reach SGCs in the bottom chamber of the transwell plate. Using these 2 approaches of labeling EVs, we found that both pre-PKH-67–labeled and newly forming PKH-67–labeled EVs could be internalized by SGCs
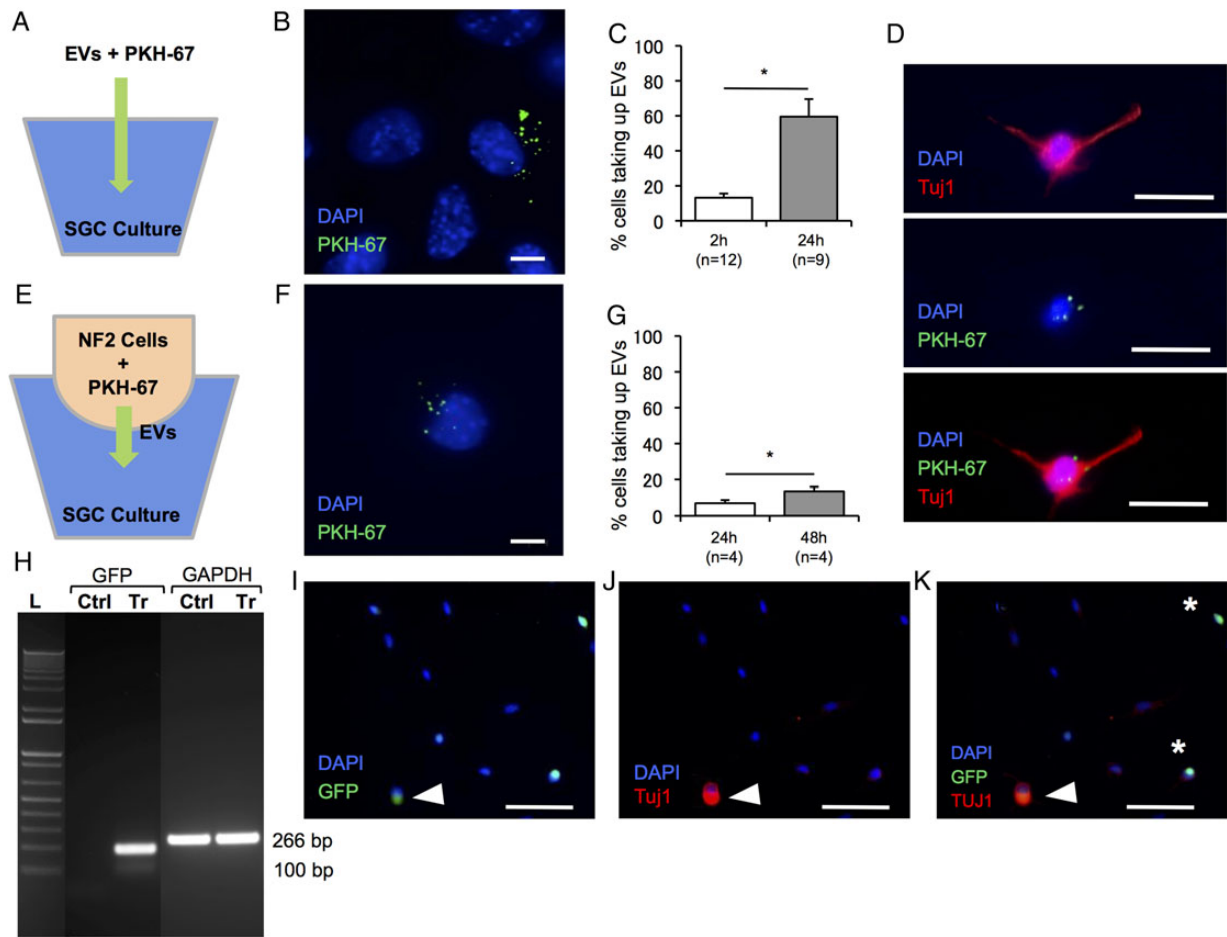
Fig. 2. EVs can transfer RNA to cochlear cells in culture, including SGCs. To study the internalization of the vesicles, (A–D) PKH-67-labeled EVs were added directly into SGC cultures or (E–G) PKH-67-labeled NF2 donor cells were cultured in a transwell plate, allowing PKH-67-labeled secreted EVs to pass through the membrane in the top chamber and reach recipient SGCs cultured in the bottom chamber. (B, F) The PKH-67-labeled EVs were identified as green cytoplasmic spots and (C, G) quantified over time (mean $\pm$ SEM, $*P < .05$). (D) A representative SGN with internalized vesicles. (H) EVs isolated from NF2 cells (lane 1) did not have the GFP mRNA, but EVs from NF2 cells transfected with pCT-CD63-GFP plasmid (lane 2) contained the GFP mRNA. L: DNA ladder. GAPDH (glyceraldehyde 3-phosphate dehydrogenase): housekeeping control mRNA for the samples in lanes 1 and 2. (I-K) Adding EVs from cells transfected with the GFP expression plasmid directly to cultured SGCs resulted in the cells expressing the GFP protein, as illustrated on a representative SGN (arrowhead). Scale bar: 2 μm (B, D, F) or 5 μm (I, J, K). DAPI, 4′,6′-diamidino-2-phenylindole.

(Fig. 2B and F, respectively). The fraction of cells taking up EVs increased from 2 h to 24 h in culture and was significantly higher for pre-PKH-67–labeled (Fig. 2C) than newly forming PKH-67–labeled EVs ($P = .001$; Fig. 2G), probably due to the higher ratio of EVs to cells in the former. SGCs internalizing EVs were identified as neurons based on immunostaining for the Tuj1 neuronal marker (Fig. 2D). The PKH-67–labeled EV-depleted media are shown in Supplementary Fig. S1A.

Extracellular Vesicles Can Transfer Tumor-Derived RNA to Cochlear Cells In vitro

Having established that SGCs can internalize tumor-derived EVs, we next tested whether SGCs could express the EVs' genetic cargo. NF2 cells were transfected with a pCT-CD63-GFP plasmid containing an expression cassette for GFP. RNA was extracted from EVs secreted by NF2 cells and reverse transcribed into cDNA. EVs from control NF2 cells that were not transfected

with the plasmid lacked the GFP mRNA (Fig. 2H, lane 1). The EVs from GFP transfected cells did contain GFP mRNA (Fig. 2H, lane 2); when these EVs were applied to cultured SGCs, GFP labeling was observed under fluorescence microscopy. GFP expression was noted in different cell types (Fig. 2I), including Tuj1-labeled neurons (Fig. 2J, arrowhead) and non-neuronal cells (Fig. 2K, asterisks).

Extracellular Vesicles from NF2 Cells Cause Damage in Cultured Cochlear Explants

Having demonstrated that dissociated SGNs can internalize NF2 cell-secreted EVs and express their genetic content, we tested whether NF2 cell-secreted EVs could also affect cochlear cells in a multilayered organotypic murine cochlear culture, referred to as a cochlear explant. Cochlear explants allow examination and quantification of hair cells and SGNs, the 2 cochlear cell types most commonly damaged in patients affected

by VSs.[13] Six microliters of EVs isolated from NF2 cell-conditioned media at a concentration of 2.5×10^{10} particles/μL were applied to cochlear explants from postnatal day 3–5 mice for 48 h; explants treated with an equal volume of PBS served as controls. Following incubation, cochlear explants were stained with anti-Myo7A antibodies to label IHC and OHC (Fig. 3A) and/or anti-Tuj1 antibodies to label neurites (Fig. 3B) and cell bodies of SGNs (Fig. 3C) and then examined using confocal microscopy. Compared with untreated explants (Fig. 3A–C, left panels), the explants treated with NF2 cell-derived EVs (Fig. 3A–C, right panels) demonstrated degenerative changes, especially at the level of neurites and SGN somata. These changes were quantified by counting the number of IHCs per 100 μm (Fig. 3D), OHCs per 100 μm (Fig. 3E), neurites per 100 μm (Fig. 3F), SGNs per 1×10^4 μm^2 (Fig. 3G), and the area of SGN somata (Fig. 3H). Control cochlear explants from different animals ($n = 7$–8) were compared with EV-treated cochlear explants from different animals ($n = 7$–8). Although the EV treatment did not affect the number of IHCs (Fig. 3D) or OHCs (Fig 3E), it significantly reduced the number of nerve fibers (Fig. 3F, $P < .05$) and neurons ($P < .05$; Fig. 3G) and the area of neuronal somata ($P < .00001$; Fig. 3H).

Cochlear Damage Caused by Extracellular Vesicles from NF2 Cells Can Be Rescued by Heparin

We further used a dual-culture system, with different concentrations of NF2 cells in the top chamber as donor cells and murine cochlear explants in the bottom chamber of a transwell plate as recipient cells, to demonstrate the dose-dependent damaging effect of NF2 cell-secreted EVs on cochlear cells (Fig. 4). Cochlear hair cells (Fig. 4A), neurites (Fig. 4B), and SGNs (Fig. 4C) were analyzed when the top chamber contained either no cells, which served as a control ($n = 11$), 1×10^6 NF2 cells ($n = 3$), 4×10^6 NF2 cells ($n = 3$), or 4×10^6 NF2 cells plus 200 μg/mL heparin ($n = 3$). Heparin is known to promote aggregation of EVs and hinder their uptake by receptor cells.[28] After 48 h in culture, the more densely plated NF2 cells (4×10^6) caused a significant loss of IHCs ($P < .04$) and OHCs ($P < .02$) compared with the control group (Fig. 4A, E, and F). There was a trend for hair cell preservation with heparin treatment (Fig. 4A), but it did not meet a significance level of $<.05$ (Fig. 4E and F). The hair cell loss was due to other toxic factors in the supernatant of NF2 culture media, such as secreted proteins, as hair cell loss was much less apparent when only purified EVs were applied (Supplementary Fig. S2). When analyzing neurites, the group with 4×10^6 NF2 cells tended to have fewer neurites—the trend that could be prevented with heparin cotreatment (Fig. 4B), albeit not significantly (Fig. 4G). The size of SGNs was significantly reduced in the groups with 1×10^6 NF2 cells and 4×10^6 NF2 cells compared with the control group (Fig. 4C, I, and J–M). There was a trend for prevention of SGN loss (Fig. 4H) and a statistically significant ($P < .05$) prevention of neuronal shrinkage with heparin cotreatment (Fig. 4C, I, and J–M).
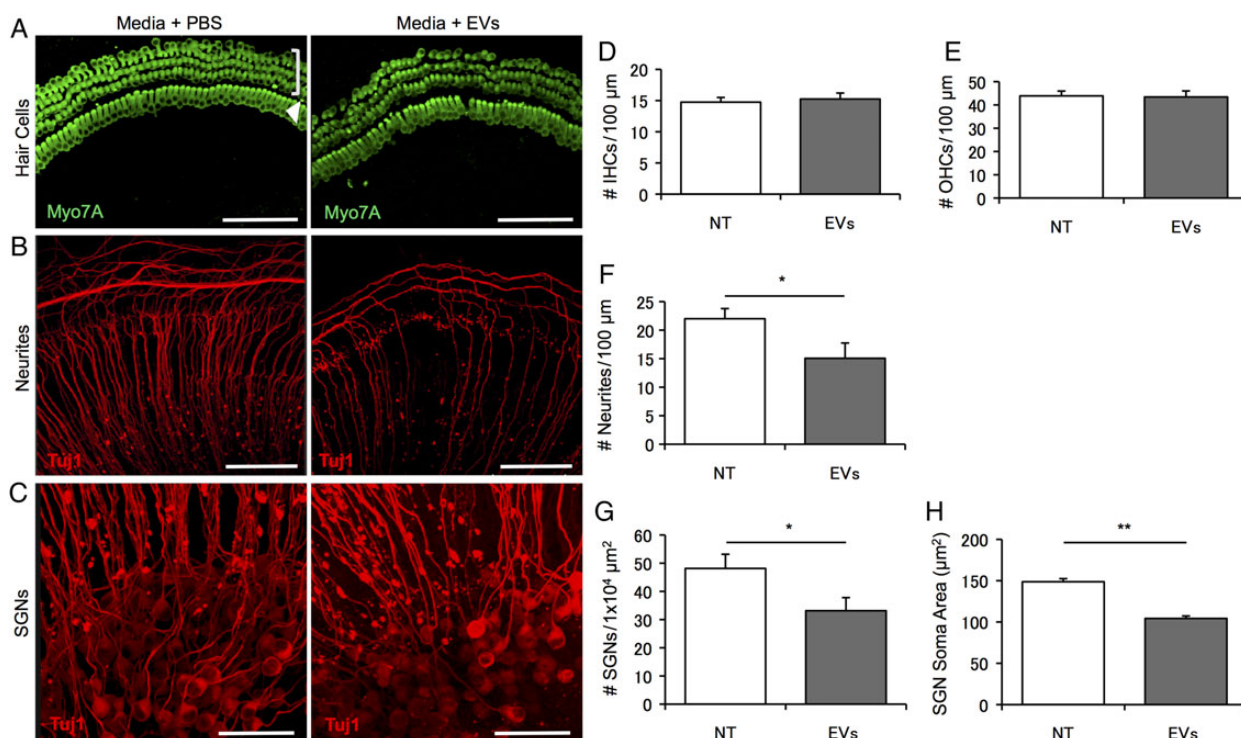

Fig. 3. EVs from NF2 cells cause damage in cultured cochlear explants. Representative images of hair cells: (A) a single row of IHCs (white arrowhead) and 3 rows of OHCs (white bracket), (B) neurites, and (C) SGNs, exposed to control PBS (6 μL) or NF2 cell-derived EVs (6 μL). Number of cells was quantified for (D) IHCs per 100 μm, (E) OHCs per 100 μm, (F) neurites per 100 μm, (G) SGNs per 1×10^4 μm^2, and (H) area of the neuronal somata for the control group (NT, no treatment) and the group treated with EVs (mean $\pm$ SEM, $n = 7$–8 explants from different animals for each in the control and EV-treated groups, *$P < .05$, **$P < .01$). Scale bar: 100 μm (A, B, C).
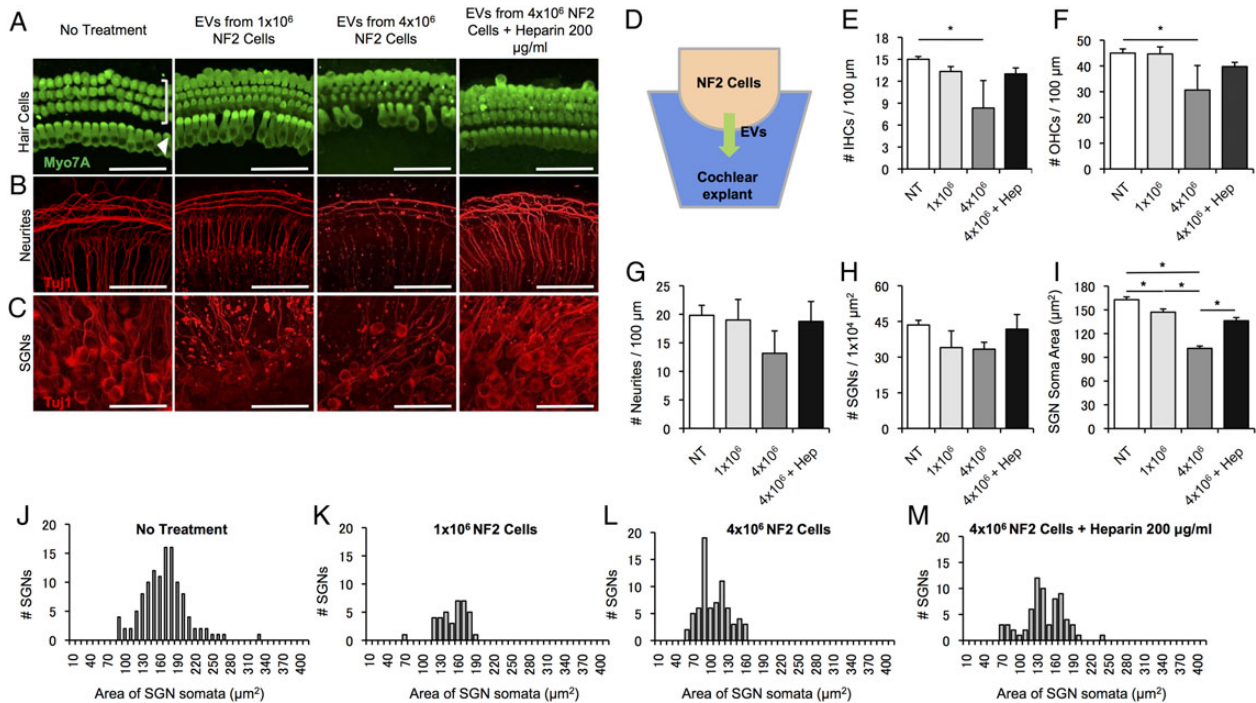
Fig. 4. Cochlear damage caused by EVs from NF2 cells can be rescued by heparin. Representative images of (A) hair cells (a single row of IHCs: white arrowhead and 3 rows of OHCs: white bracket), (B) neurites, and (C) SGNs treated with control media (no treatment), EVs from 1×10^6 NF2 cells, EVs from 4×10^6 NF2 cells, and EVs from 4×10^6 NF2 cells along with heparin in a transwell coculture system schematized in (D). Quantification of the number of IHCs per 100 μm (E), OHCs per 100 μm (F), neurites per 100 μm (G), SGNs per 1×10^4 μm² (H), and area of neuronal somata (I) for control media (NT, no treatment, white column), EVs from 1×10^6 NF2 cells (light gray column), EVs from 4×10^6 NF2 cells (dark gray column), and EVs from 4×10^6 NF2 cells along with 200 mg/mL heparin (black column) (mean ± SEM, $n = 11$ explants were used for NT controls and $n = 3$ different explants were used for all other experiments, $*P < .05$). The distribution of the area of SGN somata in cochlear explants treated with control media (no treatment) (J), EVs from 1×10^6 NF2 cells (K), EVs from 4×10^6 NF2 cells (L), and EVs from 4×10^6 NF2 cells along with 200 mg/mL heparin (M). The data in panels (J)–(M) are summarized in the panel (I). Scale bar: 50 μm (A, C) or 100 μm (B). Hep, heparin.

Extracellular Vesicles from Vestibular Schwannomas Associated with Poor Hearing Damage Cochlear Cells

Having established that EVs derived from the NF2 cell line can damage cochlear cells in an explant model (Figs 3 and 4), we next tested whether EVs derived from primary human VSs could have a similar effect. Primary VSs can be associated with GH in the ipsilateral ear, defined as word recognition better than 70% out of a possible 100% and pure tone average <30 dB hearing level, or PH. This classification system was developed by the American Academy of Otolaryngology–Head and Neck Surgery.[29] We have previously demonstrated that primary cultured VS cells are representative of their parent tumors.[20] We applied EVs from conditioned media of cells from 3 VSs from patients with GH, using an average concentration ± SEM of $1.9 \times 10^{10} \pm 0.6$ particles/μL, and from 3 VSs from patients with PH in the ipsilateral ear, with an average concentration of $2.8 \times 10^{10} \pm 0.9$ particles/μL. Patient demographics are summarized in Table 1 and Supplementary Fig. S3. Individual audiograms for all patients are shown in Supplementary Fig. S4. The tumors in the GH and PH group were matched with respect to patients' age, sex, and tumor size. Moreover, the size distribution of the EVs and concentration of particles were found to be similar between EVs from GH ($n = 3$) and PH ($n = 3$) VSs ($P > .05$). The EVs from cells cultured from each tumor were applied to 3–4 different cochlear explants, for a total of 10 different explants for the GH VS group and 10 different explants for the PH VS group (Fig. 5); cochlear explants from 10 different animals served as no-treatment controls. Compared with no-treatment controls (Fig. 5A, left panel), explants treated with EVs from VSs associated with good (Fig. 5A, middle panel) or poor hearing (Fig. 5A, right panel) did not lose IHCs (Fig. 5E) or OHCs (Fig. 5F). However, the number of neurites (Fig. 5B and G) and SGNs (Fig. 5C and H) as well as the area of SGN somata (Fig. 5I) were significantly reduced in explants treated with EVs from VSs associated with PH ($P = .04$, $P < .0001$, and $P < .0001$, respectively) compared with the control group, but not in explants treated with EVs from VSs associated with GH. Individual measurements in Fig. 5I are depicted as the distribution of the area of SGN somata in Fig. 5K–M. Using the Poly Caspase kit to mark apoptotic cells with a green stain (Fig. 5D), we found a significantly greater number of apoptotic SGNs in cochlear explants treated with EVs from cultured VSs associated with PH than in control explants ($P = .015$) or in explants treated with EVs from cultured VSs associated with GH ($P = .022$; Fig. 5J). The number of counted SGNs was 149, 123, and 162, respectively, which was not statistically significant among the groups.

Table 1. Patient demographics

Tumor ID	Age	Gender	Side	Tumor Size (mm)	Hearing Level			
					Ipsilateral Ear		Contralateral Ear	
					PTA (dB)	WR (%)	PTA (dB)	WR (%)
VS 1	55	F	Right	22	12	96	8	98
VS 2	46	F	Right	24	12	94	10	100
VS 3	49	F	Left	12	22	84	6	94
VS 4	**45**	**F**	**Left**	**12**	**58**	**20**	**2**	**100**
VS 5	**53**	**F**	**Right**	**29**	**35**	**94**	**18**	**100**
VS 6	**61**	**F**	**Right**	**20**	**40**	**20**	**15**	**100**

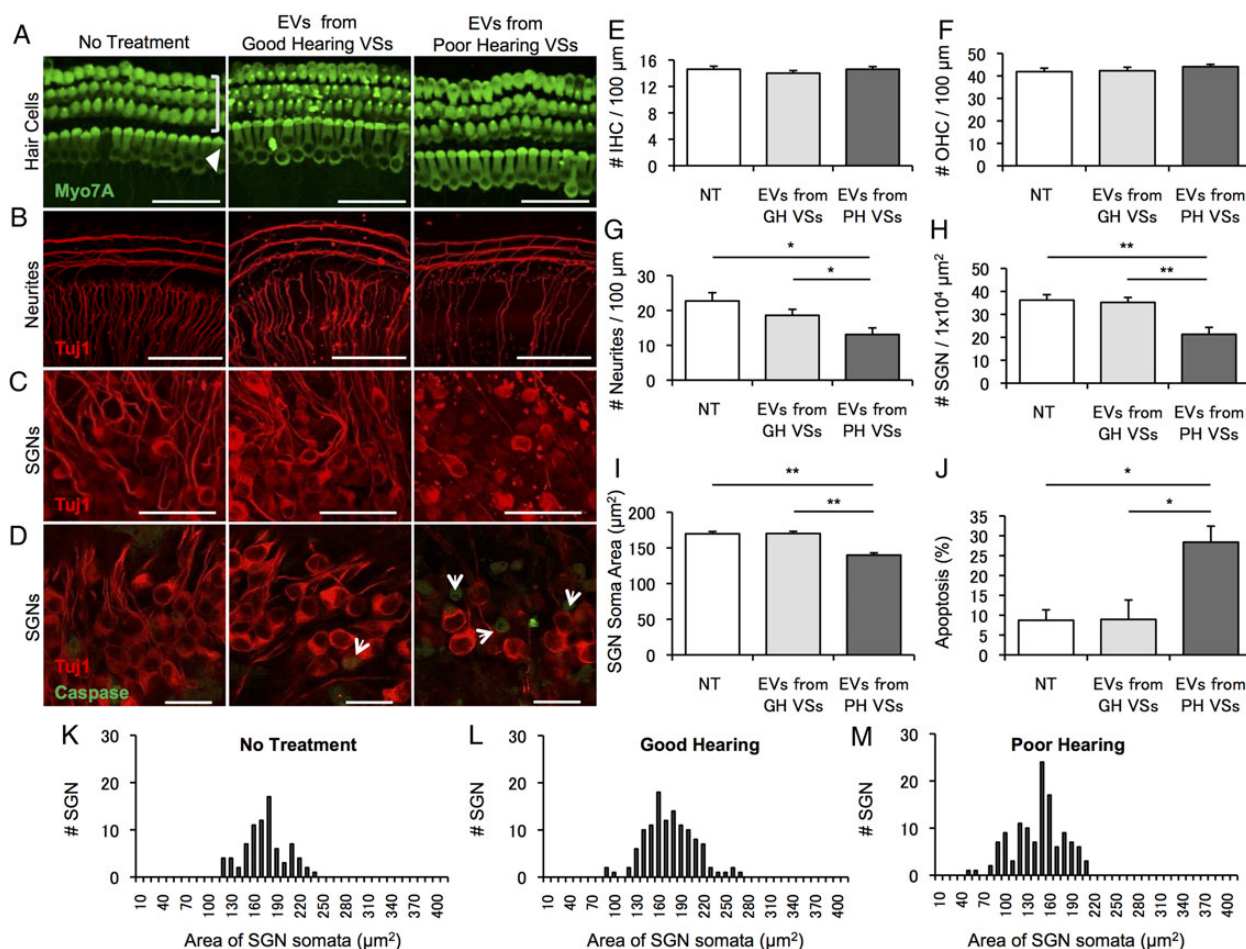Abbreviations: PTA, pure tone average; WR, word recognition. Poor hearing patients in bold font.

Fig. 5. Application of isolated EVs from VSs associated with PH causes cochlear explant damage. Representative images of (A) hair cells (a single row of IHCs [white arrowhead] and 3 rows of OHCs [white bracket]), (B) neurites, (c) SGNs, and (D) SGNs labeled with polycaspase stain to identify apoptotic cells treated with control media (NT, no treatment; 6 μL of PBS), isolated EVs (6 μL of 2×10^{10} particles/μL) from a VS associated with GH, and extracted EVs (6 μL of 2.9×10^{10} particles/μL) from a VS associated with PH are shown. Quantification of the number of (E) IHCs per 100 μm, (F) OHCs per 100 μm, (G) neurites per 100 μm, (H) SGNs per 1×10^4 μm², (I) area of neuronal somata, and (J) apoptosis noted in SGN for control media (NT, no treatment, white column), isolated EVs from a VS associated with GH (light gray column), and isolated EVs from a VS associated with PH (dark gray column) (mean ± SEM, *$P < .05$, **$P < .01$). $n = 10$ explants from different animals for each of 3 groups (E–I), except for (J) apoptosis assay where $n = 3$ or 4 explants from different animals for the NT, the GH, and the PH VS groups. The distribution of the area of SGN somata in cochlear explants treated with control media (no treatment) (K), isolated EVs from a VS associated with GH (L), and isolated EVs from a VS associated with PH (M). The data in panels (K)–(M) are summarized in panel (I). Scale bar: 25 μm (D), 50 μm (A, C) or 100 μm (B).

Discussion

Our study is the first to isolate EVs produced by cultured primary VS cells and a cultured NF2 VS cell line. In order to elucidate the function of EVs from VSs, we further assessed whether VS-secreted EVs could cause cochlear damage. Using a cochlear explant culture model and a transwell coculture system, we demonstrated that SGNs are most susceptible to this damage; hair cells are also vulnerable, especially to EVs secreted by NF2 VS cells. Importantly, EVs secreted by VSs associated with PH, but not EVs secreted by VSs associated with GH, produced a damaging effect on the SGNs.

VS secretions provide an alternative and complementary mechanism to mechanical compression of the cochlear nerve in mediating VS-induced hearing loss. The neurotoxic and ototoxic components of the VS secretome could reach the adjacent cochlea via the fundus of the internal auditory canal to affect cochlear function. Our focus on VS secretions is motivated by clinical observations that cannot be simply explained by cochlear nerve compression. Specifically, there is no correlation between the tumor extent within the internal auditory canal or radiographic tumor size and audiometric threshold shifts in people with sporadic VSs.[14,15] In addition, VS patients can develop sudden or progressive audiometric threshold shifts despite the lack of VS growth.[15] A role for VS secretions in SNHL is supported by the observations that total levels of protein in cochlear fluids of VS patients are 5–15 times higher than in healthy individuals[30,31] and that the proteome of cochlear fluid is different in VS patients compared with those without the tumor.[32] We have found that VSs associated with GH secrete higher levels of fibroblast growth factor 2 than VSs associated with PH, irrespective of the tumor size,[33] while VS secretion of tumor necrosis factor alpha correlates with poor hearing.[34] Moreover, our cDNA microarray study of VSs stratified by hearing has revealed differences in gene expression profiles between VSs that do and those that do not cause hearing loss.[18] Another cDNA microarray study reported that expression levels of platelet derived growth factor–A gene inversely correlated with SNHL in VS patients.[35] The apparent intrinsic genetic differences in VSs that do versus those that do not cause SNHL are likely to be reflected in VS-derived EVs, since EVs contain the genetic profile of their cell of origin.[36] It is likely that both EVs and free soluble molecules in VS secretions modulate cochlear function. An advantage of EVs is that they can concentrate donor cell–specific cargo.[37] For future clinical application, it will be important to identify the specific factors in EVs responsible for the cochlear damage noted in our study. Identification analyses such as next-generation microRNA sequencing and proteomic analysis of VS-released EVs could provide insight into the molecular pathways leading to hearing loss due to VSs.

Conclusions

Our findings strongly motivate future work to identify the EV-derived cargo mediating cochlear damage, as this could provide insight into much needed prognostic and therapeutic targets for prevention and treatment of hearing loss due to VSs and potentially other causes. Understanding mechanisms of hearing loss due to VS, such as by VS-secreted EVs, can expedite the path to pharmacotherapies against this common, debilitating symptom of VS for which approved nonsurgical therapies do not exist.

Supplementary material

Supplementary material is available at *Neuro-Oncology Journal* online (http://neuro-oncology.oxfordjournals.org/).

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References

1. Andaloussi SE, Mäger I, Breakefield XO, et al. Extracellular vesicles: biology and emerging therapeutic opportunities. *Nat Rev Drug Discov*. 2013;12(5):347–357.

2. Valadi H, Ekstoröm K, Bossios A, et al. Exosome-mediated transfer of mRNAs and microRNAsis a novel mechanism of genetic exchange between cell. *Nat Cell Biol*. 2007;9(6):654–659.

3. Mizrak A, Bolukbasi MF, Ozdener GB, et al. Genetically engineered microvesicles carrying suicide mRNA/protein inhibit schwannoma tumor growth. *Mol Ther*. 2013;21(1):101–108.

4. Skog J, Würdinger T, van Rijin S, et al. Glioblastoma microvesicles transport RNA and proteins that promote tumor growth and provide diagnostic biomarkers. *Nat Cell Biol*. 2008;10(12): 1470–1476.

5. Mahaley MS Jr, Mettlin C, Natarajan N, et al. Analysis of patterns of care of brain tumor patients in the United States: a study of the brain tumor section of the AANS and the CNS and the commission on cancer of the ACS. *Clin Neurosurg*. 1990;36: 347–352.

6. Robbins PD, Morelli AE. Regulation of immune responses by extracellular vesicles. *Nature Rev Immunol*. 2014;14(3):195–208.

7. Fiandaca MS, Kapogiannis D, Mapstone M, et al. Identification of preclinical Alzheimer's disease by a profile of pathogenic proteins in neurally derived blood exosomes: a case-control study. *Alzheimers Dement*. 2015;11(6):600–607.

8. Kordelas L, Rebmann V, Ludwig AK, et al. MSC-derived exosomes: a novel tool to treat therapy-refractory graft-versus-host disease. *Leukemia*. 2014;28(4):970–973.

9. Akers JC, Gonda D, Kim R, et al. Biogenesis of extracellular vesicles (EV): exosomes, microvesicles, retrovirus-like vesicles, and apoptotic bodies. *J Neurooncol*. 2013;113(1):1–11.

10. Neff BA, Welling DB, Akhmametyeva E, et al. The molecular biology of vestibular schwannomas: dissecting the pathogenic

process at the molecular level. *Otol Neurotol*. 2006;27(2): 197–208.

11. Matthies C, Samii M. Management of 1000 vestibular schwannomas (acoustic neuromas): clinical presentation. *Neurosurg*. 1997;40(1):1–9.

12. Lee JD, Lee BD, Hwang SC. Vestibular schwannoma in patients with sudden sensorineural hearing loss. *Skull Base*. 2011;21(2): 75–78.

13. Roosli C, Linthicum FH Jr, Cureoglu S, et al. Dysfunction of the cochlea contributing to hearing loss in acoustic neuromas: an underappreciated entity. *Otol Neurotol*. 2012;33(3):473–480.

14. Nadol JB Jr, Diamond PF, Thornton AR. Correlation of hearing loss and radiologic dimensions of vestibular schwannomas (acoustic neuromas). *Am J Otol*. 1996;17(2):312–316.

15. Caye-Thomasen P, Dethloff T, Hansen S, et al. Hearing in patients with intracanalicular vestibular schwannomas. *Audiol Neurootol*. 2007;12(1):1–12.

16. Asthagiri AR, Vasquez RA, Butman JA, et al. Mechanisms of hearing loss in neurofibromatosis type 2. *PLoS One*. 2012;7(9): e46132.

17. Rouleau GA, Wertelecki W, Haines JL, et al. Genetic linkage analysis of bilateral acoustic neurofibromatosis to a DNA marker on chromosome 22. *Nature*. 1987;329(6136):246–248.

18. Stankovic KM, Mrugala MM, Martuza RL, et al. Genetic determinants of hearing loss associated with vestibular schwannomas. *Otol Neurotol*. 2009;30(5):661–667.

19. Hung G, Li X, Faudoa R, et al. Establishment and characterization of a schwannoma cell line from a patient with neurofibromatosis 2. *Int J Oncol*. 2002;20(3):475–482.

20. Dilwali S, Patel PB, Roberts DS, et al. Primary culture of human Schwann and schwannoma cells: improved and simplified protocol. *Hear Res*. 2014;315:25–33.

21. Kao SY, Kempfle JS, Jensen JB, et al. Loss of osteoprotegerin expression in the inner ear causes degeneration of the cochlear nerve and sensorineural hearing loss. *Neurobiol Dis*. 2013;56: 25–33.

22. Parker M, Brugeaud A, Edge AS. Primary culture and plasmid electroporation of the murine organ of Corti. *J Vis Exp*. 2010;36: e1685.

23. Haque KD, Pandey AK, Kelley MW, et al. Culture of embryonic mouse cochlear explants and gene transfer by electroporation. *J Vis Exp*. 2015;95:e52260.

24. Ekstrom K, Valadi H, Sjostrand M, et al. Characterization of mRNA and microRNA in human mast cell-derived exosomes and their

transfer to other mast cells and blood CD34 progenitor cells. *J Extracell Vesicles*. 2012;1. doi: 10.3492/jev.v1i0.18389.

25. Wei L, Ding D, Salvi R. Salicylate-induced degeneration of cochlea spiral ganglion neurons-apoptosis signaling. *Neuroscience*. 2010; 168(1):288–299.

26. Vlassov AV, Magdaleno S, Setterquist R, et al. Exosomes: current knowledge of their composition, biological functions, and diagnostic and therapeutic potentials. *Biochim Biophys Acta*. 2012;1820(7):940–948.

27. Zaborowski JP, Balaj L, Breakefield XO, et al. Extracellular vesicles: composition, biological relevance and methods of study. *BioScience*. 2015;65:783–794.

28. Atai NA, Balaj L, van Veen H, et al. Heparin blocks transfer of extracellular vesicles between donor and recipient cells. *J Neurooncol*. 2013;115(3):343–351.

29. Committee on hearing and equilibrium guidelines for the evaluation of hearing preservation in acoustic neuroma (vestibular schwannoma): committee on hearing and equilibrium. *Otolaryngol Head Neck Surg*. 1995;113:179–180.

30. Silverstein H. A rapid protein test for acoustic neurinoma. *Arch Otolaryngol*. 1972;95(3):202–204.

31. Silverstein H. Labyrinthine tap as a diagnostic test for acoustic neurinoma. *Otolaryngol Clin North Am*. 1973;6(1):229–244.

32. Lysaght AC, Kao SY, Paulo JA, et al. Proteome of human perilymph. *J Proteome Res*. 2011;10(9):3845–3851.

33. Dilwali S, Lysaght A, Roberts D, et al. Sporadic vestibular schwannomas associated with good hearing secrete higher levels of fibroblast growth factor 2 than those associated with poor hearing irrespective of tumor size. *Otol Neurotol*. 2013; 34(4):748–754.

34. Dilwali S, Landegger LD, Soares VY, et al. Secreted factors from human vestibular schwannomas can cause cochlear damage. *Sci Rep*. 2015;5:18599.

35. Lassaletta L, Martinez-Glez V, Torres-Martin M, et al. cDNA microarray expression profile in vestibular schwannoma: correlation with clinical and radiological features. *Cancer Genet Cytogenet*. 2009;194(2):125–127.

36. Jenjaroenpun P, Kremenska Y, Nair VM, et al. Characterization of RNA in exosomes secreted by human breast cancer cell lines using next-generation sequencing. *PeerJ*. 2013;5(1):e201.

37. Chen C, Skog J, Hsu CH, et al. Microfluidic isolation and transcriptome analysis of serum microvesicles. *Lab Chip*. 2010; 10(4):505–511.

Correlation Between Aspirin Intake and Reduced Growth of Human Vestibular Schwannoma

Volumetric Analysis

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Objective: To determine whether people with sporadic vestibular schwannoma (VS) who take aspirin for unrelated medical reasons exhibit less tumor growth than nonaspirin users. We previously demonstrated the efficacy of salicylates in inhibiting VS growth in vitro, corroborating the results of our retrospective clinical study, which found halted VS growth (based on linear tumor measurements) in aspirin users. The current study evaluates this association using more accurate tumor volumetric measurements, and quantifies the degree of frequency-specific, VS-induced hearing loss.
Study Design: Retrospective analysis.
Setting: Tertiary care hospital.
Patients: Diagnosed with VS between 1980 and 2013, followed by serial magnetic resonance imaging for at least 1 year.
Main Outcome Measures: Patient history of aspirin intake; change in VS volume over time of observation; frequency-specific, VS-induced audiometric threshold shifts.

Results: Of the 347 patients followed by serial magnetic resonance imaging scans, 86 had sequential scans available for 3D-segmented volumetric analysis for up to 11 years of follow-up (median 53 mo). Twenty-five (29%) had documented history of aspirin intake; 8 (32%) of these demonstrated VS growth. Of the 61 (71%) nonusers, 36 (59%) demonstrated growth. A significant inverse association was found among aspirin users and VS growth: odds ratio 0.32, 95% confidence interval 0.11 to 0.91. VS-induced audiometric thresholds shifts were larger above than below 2000 Hz.
Conclusion: Our volumetric analysis of VS growth reaffirms the results of our linear analysis and suggests that aspirin may inhibit VS growth. The audiometric findings are consistent with the previously reported VS-induced predominantly high-frequency sensorineural hearing loss. **Key Words:** Aspirin intake—Magnetic resonance imaging—Sporadic vestibular schwannoma—Volumetric analysis.

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Vestibular schwannoma (VS) is one of the most common nonmalignant intracranial nerve sheath tumors (1). VS accounts for 8% of all intracranial tumors and 80% of cerebellopontine angle (CPA) tumors. VS typically arises from Schwann cells of the superior and inferior vestibular nerves that are part of the VIII cranial nerve. The tumor forms sporadically and unilaterally in 95% of patients

(incidence 1:10,000) (2) or in association with neurofibromatosis type 2 whose hallmark is bilateral VSs (incidence 1:33,000) (3). VS incidence has increased over the years, presumably because of improved imaging techniques and diagnostic awareness among clinicians (4,5). Contrast-enhanced magnetic resonance imaging (MRI) of the brain is currently the gold standard for diagnosing VS. The widespread use of brain MRI has enabled discovery of incidental and asymptomatic VSs (5–7). Nevertheless, many people with small VS may remain undiagnosed, making it difficult to determine the prevalence of VS in the general population. When serially sectioned temporal bones were routinely examined in 521 individuals, the histologic incidence of VS was 1:100 (8), suggesting that VS is much more common than clinically recognized.

Sensorineural hearing loss is the most commonly presenting sign of VS, occurring in 95% of patients

(9). Other common symptoms include tinnitus and dizziness. As VSs grow, they can cause additional cranial nerve neuropathies, hydrocephalus, and even death from brainstem compression (9,10). Current management options for growing or symptomatic VSs are essentially limited to stereotactic radiation therapy and surgical resection via craniotomy. Both of these options involve significant potential risks, including deafness and facial paralysis. Identifying effective pharmacotherapies for VS is a major unmet medical need as no drug is FDA-approved to treat VS. The only drug showing activity against VS in clinical trials is bevacizumab, which inhibits vascular endothelial growth factor. Bevacizumab decreases tumor growth or improves hearing, typically temporarily, in 54% of patients (11). However, long-term use of this expensive drug is associated with severe side effects, including renal failure (12). New and well-tolerated therapies against VSs are therefore urgently needed.

To address this need, we have been investigating salicylates, a class of nonsteroidal antiinflammatory drugs that are well tolerated and inexpensive. We have focused specifically on aspirin because it modifies the COX-2's enzymatic activity (13) and COX-2 expression in VS correlates with a high tumor proliferation rate (14). Moreover, aspirin provides chemoprevention for multiple human malignancies, including colon, gastric, breast, and prostate cancer (15–18) by modulating COX-2 activity. Our retrospective longitudinal clinical study of VS patients who underwent serial MRI scanning revealed that patients taking aspirin for unrelated medical reasons were less likely to have VS growth than patients not taking aspirin (19). This study, based on linear VS measurements, inspired a mechanistic study with primary human VS cells (20). In the latter study we found that COX-2 is aberrantly expressed in human VSs and their derived primary VS cultures relative to control human nerve specimens and their derived primary Schwann cells (SCs). Primary VS culture proliferation rate correlated with their secretion of prostaglandin E2, the downstream enzymatic product of COX-2. Aspirin was cytostatic against cultured VS cells and did not affect healthy SCs (20).

Moreover, our bioinformatic network analysis of all genes reported to be differentially expressed in human VS revealed a pro-inflammatory transcription factor nuclear factor-kappa B (NF-κB) as a central molecule in VS pathobiology (21). This finding was validated by demonstrating aberrant activation of the canonical NF-κB complex in human VSs and derived VS cultures relative to control nerves and SCs, and by proving cytotoxicity when directly targeting NF-κB in VS cells (21). Importantly, aspirin is known to inhibit NF-κB activation (22,23).

Motivated by our preclinical (20) and clinical (19) data implicating aspirin's potential to prevent VS growth, we undertook the current study to define the correlation between aspirin intake and VS volume. Volumetric tumor measurements are more precise than linear tumor measurements (24–27) based on Response Evaluation Criteria in Solid Tumors (RECIST) (28). The volumetric analysis reaffirmed the results of our linear analysis, suggesting that aspirin may inhibit VS growth.

METHODS

Patient Cohort

The study included patients diagnosed with sporadic VS at Massachusetts Eye and Ear Infirmary (MEEI) from January 1, 1980 to April 1, 2012. The final patient population included 86 of our originally described cohort of 347 patients followed by serial MRI (19) for at least 1 year; this subset of patients had all sequential scans available for 3D-segmented volumetric analysis. Patients were grouped into "aspirin users" and "nonusers" of aspirin. Aspirin users had documented history of aspirin intake and at least one comorbidity that warranted long-term aspirin use or met the age criterion for aspirin prophylaxis against myocardial infarction (age 45 or older in men) or stroke (age 55 or older in women) (29). The study was approved by the institutional review board (HSC protocol number: 12-036H, P.I.: K.M.S).

Quantifying Tumor Volume

Segmented 3D volumetric analysis of VS was performed for each patient on axial contrast-enhanced T1-weighted magnetic resonance images using a Voxar unit (Toshiba Medical Systems) (30). For each study, the tumor was manually outlined in the axial plane on every third sequential section. A computerized compilation of the measured slice's volume and an assessment of total tumor volume were used to create a segmented volumetric model (27). Tumor volumes were expressed in cubic centimeters (cc). The first of the serial MRI scans was defined as baseline. In our original study (19) investigating changes in VS's single linear dimension on serial MRI scans, tumors were categorized as growing if the increase in size was greater than 0 cm, and not growing if they were stable or shrinking. The criterion for tumor growth was redefined for the present study because of the well-documented error associated with tumor volume remeasurement (24,26,31–35) and up to 50% remeasurement variability in very small tumors (volumes less than 0.05 cc)_ENREF_39. Therefore, for tumors whose original volume was >0.05 cm, we defined tumor growth as >20% positive change in volume from the first MRI scan. "No-growth" was defined as any change in tumor volume <20%, including tumor shrinkage, for tumors whose original volume was ≥0.05 cc and any change in tumor volume for tumors whose original volume was less than 0.05 cc (n = 3 aspirin users and n = 2 nonusers). All measurements were made using the same algorithm and computer system by one experienced investigator (C.K.K.) who was blinded to aspirin use. The categorical classification of growth versus no growth was used because VS growth rates varied substantially among tumors.

Statistical Analysis

STATA (version 12.1, StataCorp, College Station, TX) was used for data analysis.

Fisher's exact test was used to compare the difference in proportion of VS demonstrating growth versus no growth between aspirin users and nonusers and to calculate the odds ratio (OR) and the corresponding 95% confidence interval (CI); $p < 0.05$ was considered significant. Multiple logistic regression was used to determine if age and sex were potential

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TABLE 1. *Summary of vestibular schwannoma patients included in the study*

	Patients With Serial MRI Scans Identified in Original Study[17]	Patients With Serial MRI Scans Available for 3D Volumetric Analysis (Follow-up >1 yr)	Tumor Growth[a]	No Tumor Growth
Aspirin users	81	25	8	17
Nonusers	266	61	36	25
Age-adjusted nonusers[b]	222	57	34	23

[a]Tumor growth defined as >20% change in tumor volume for tumors whose initial volume was at least 0.05 cc.
[b]Age adjustment was performed by eliminating patients from the nonusers so that the aspirin users and age-adjusted nonusers are not statistically significant in age.
MRI indicates magnetic resonance imaging.

confounders. Using an independent sample t test (with $p < 0.05$ considered significant), aspirin users, nonusers, and age-adjusted nonusers were compared with regard to tumor volume at the time of the first MRI scan (null hypothesis H_0: mean tumor volumes are equal).

Audiometric Analysis

Audiometric thresholds were obtained at 250, 500, 1000, 2000, 4000, and 8000 Hz using the standard protocol (36). To quantify the degree of VS-induced hearing loss, the difference in audiometric thresholds (in dB) between the ear ipsilateral to the VS and the contralateral ear was calculated for each frequency and each patient. Differences between aspirin users and nonusers were compared using t test at every frequency. Benjamini–Hochberg correction for multiple hypotheses was applied to generated p values and Benjamini–Hochberg corrected p values ≤ 0.05 were considered significant. VS-induced thresholds shifts across frequencies were analyzed using analysis of variance (ANOVA).

RESULTS

Of the 81 aspirin users and 266 nonusers with serial MRI scans originally identified in our linear study of VS growth (19), scans from a total of 86 patients followed for at least 1 year were available for 3D volumetric analysis (Table 1). Of the 86 available patients for the study, 25 were identified as aspirin users and 61 as nonusers. Among the aspirin users, 11 patients took 81 mg (baby strength) daily and 14 patients did not have the aspirin dose specified; the dose is assumed to be 81 or 325 mg daily based on the common medical usage. There was no mention of high-dose aspirin use in the medical records (19). Cardiovascular disease was the most documented associated comorbidity, reported in 18 patients; seven patients had no documented associated comorbidity, except age which qualified them for aspirin prophylaxis. VS growth was noted in 44 patients (51%), 8 (18%) of whom were aspirin users (Table 1). Forty-two patients (49%) showed no tumor growth; 17 (40%) were aspirin users. The difference in these categorical variables, i.e., VS growth versus no growth between aspirin users and nonusers was statistically significant ($p = 0.03$; OR: 0.32; 95% CI; 0.11–0.91; Table 2) and not confounded by age ($p = 0.4$) or sex ($p = 0.26$).

Because VS growth rates can be inversely correlated with age (37), we performed an age-adjustment among

the nonusers by selecting the nonusers whose ages closely matched the age of aspirin users. The average age of aspirin users (67.2 yr) was significantly different ($p = 0.02$) from the average age of nonusers (60.4 yr), but not significantly different from the average age for age-adjusted nonusers (62.7 yr, $p = 0.08$, Fig. 1A). Sex distribution within and across groups is shown in Figure 1B. There were more women among the nonusers and more men among aspirin users, consistent with the reports that women are less likely to take aspirin regularly than men (38). The MRI follow-up time ranged from 13 to 132 months, with the mean ± standard error of the mean being 4.5 ± 0.3 years for nonusers, 4.4 ± 0.3 years for age-adjusted nonusers and 4.7 ± 0.5 years for aspirin users (Fig. 1C). The average tumor volume (cc) at the time of the first MRI, expressed as mean ± standard error of the mean, was 0.83 ± 0.18, 0.9 ± 0.19, and 0.77 ± 0.25 for nonusers, age-adjusted nonusers, and aspirin users, respectively. This trend for smaller tumor volume among the aspirin users did not meet our criterion for significance.

The degree of VS-induced hearing loss was quantified by calculating the audiometric threshold shift between the ear ipsilateral to the VS and the contralateral ear for patients with available audiograms (Fig. 2). The initial audiogram, at the time of VS diagnosis, and the final available audiogram were analyzed. The average VS-induced threshold shift between aspirin users (n = 17)

TABLE 2. *Analysis of VS growth versus no growth among aspirin users and nonusers*

Comprehensive Analysis

	VS Growth	No VS Growth	OR (95% CI)
Aspirin users	8	17	0.32 (0.11–0.91)
Nonusers	36	25	1

VS growth was noted in 44 patients (51%), 8 (18%) of whom were aspirin users. Forty-two patients (49%) showed no tumor growth; 17 (40%) were aspirin users. The difference in these categorical variables, i.e., VS growth versus no growth between aspirin users and nonusers was statistically significant ($p = 0.03$; OR: 0.32; 95% CI; 0.11–0.91; Table 2) and not confounded by age ($p = 0.4$) or gender ($p = 0.26$).
CI indicates confidence interval; OR, odds ratio; VS, sporadic vestibular schwannoma.
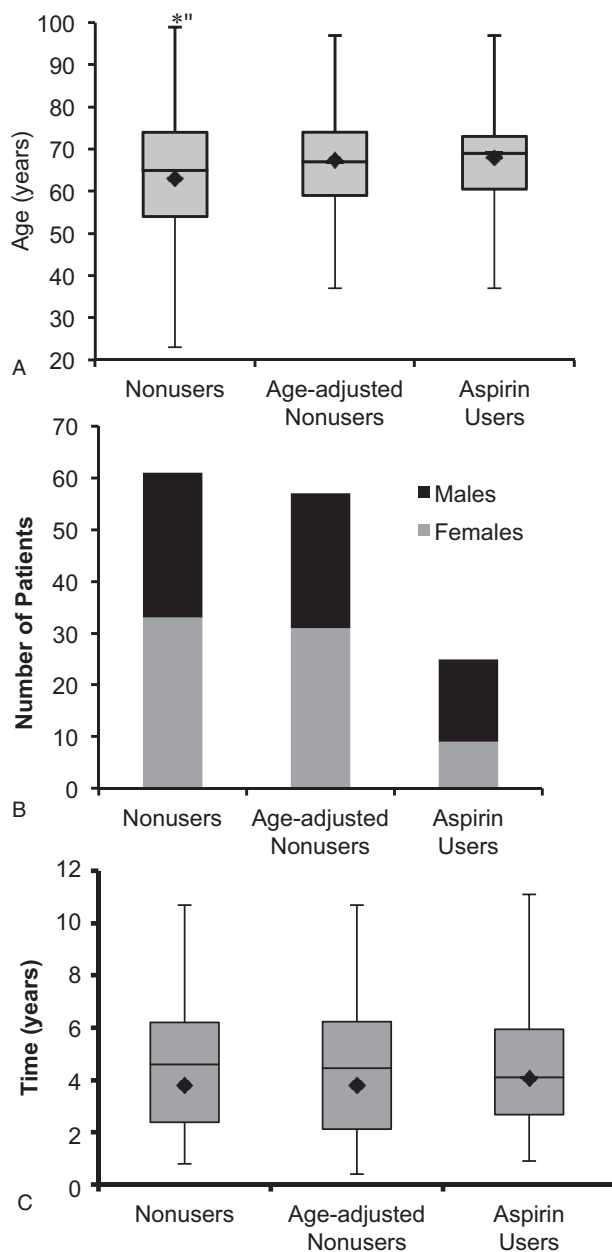
FIG. 1. Patient demographics. *A*, Age distribution (yr) for nonusers, age-adjusted nonusers, and aspirin users. The box plot represents quartile 1, median, and quartile 3. The black diamond represents the corresponding mean for each group. Error bars represent minimum and maximum values for each cohort. *p<0.05 for nonusers in comparison to aspirin users. *B*, Cohort sizes stratified by gender for nonusers (n = 61), age-adjusted nonusers (n = 57), and aspirin users (n = 25). *C*, Distribution of MRI follow-up time (in years) was not significantly different between nonusers and aspirin users. Symbols for the box plot as in (*A*). MRI indicates magnetic resonance imaging.

FIG. 2. VS-induced threshold shift, calculated as the difference in audiometric thresholds between the ear ipsilateral to the VS and the contralateral ear, shows frequency dependence. The average VS-induced threshold shift is shown for aspirin users (n = 17) and nonusers (n = 40). Error bars represent ± SEM. VS indicates vestibular schwannoma.

and nonusers (n = 40) was not statistically significant at any frequency on initial and final audiograms after applying Benjamini–Hochberg correction for multiple comparisons. The observed trend for larger thresh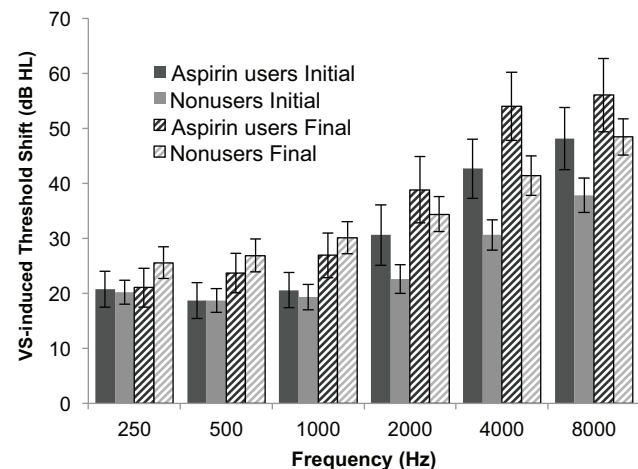olds shifts at higher frequencies, above 2000 Hz, than lower frequencies was statistically significant for both aspirin users ($p = 0.0002$ on initial audiogram, $p = 0.0285$ on final audiogram) and nonusers ($p = 0.0002$ on initial audiogram and $p = 0.0035$ on final audiogram). The increase in VS-induced hearing loss over time is consistent with the reports that hearing loss typically worsens in VS patients with time, even if there is no measurable VS growth (39,40).

DISCUSSION

We used 3D volumetric analysis to reaffirm the inverse association between aspirin intake and VS growth that we had previously observed using linear analysis (19). Previous studies have established that 3D volumetric analysis is superior to linear analysis for estimating tumor size and growth (24–27).

Although VS growth rate among aspirin users in our population was higher than VS growth rates reported in other studies, others did not specifically classify patients based on aspirin use. In a series involving 114 patients, Fayad et al. (41) reported 38% growth rate based on linear tumor measurements. In a smaller study involving 15 patients, Niemczyk et al. (24) reported 43% growth rate in unilateral VSs when using volumetric analysis. Volumetric tumor analysis is known to identify higher growth rates than linear analysis: when analyzing 24 unilateral VSs, Walz et al. (27) identified progression in 75% more patients with segmented volumetric analysis than with maximum linear dimension measurement. Because VSs assume complex configurations, linear measurements can inaccurately estimate tumor volume and growth compared with volumetric analysis (27). Taken together, our results motivate future measurements of volumetric tumor growth in large

cohorts of VS patients with carefully documented intake of antiinflammatory medications.

Although information regarding aspirin use patterns is incomplete in our study, it is likely that patients took 81 or 325 mg daily chronically and not only occasionally. Our in vitro study with primary human VS cells showed a significant reduction in VS cell proliferation when salicylate levels of 3.3 mg/dL were measured in the media of cells treated with 5 mM aspirin (20). To reach similar salicylate concentrations in serum, a daily dose of about 800 mg aspirin would be needed (42). This dose is well below the range of salicylate toxicity, which starts to become apparent when serum salicylate levels exceed 25 mg/dL (43). However, aspirin concentrations that affect cultured VS cells cannot be directly scaled to predict concentrations required to achieve a therapeutic effect with systemic administration. In a study investigating aspirin's role in preventing colorectal cancer, daily aspirin use at any dose was found to be effective (44). Specifically, aspirin treatment at a dose ranging from 75 to 1,200 mg/day was associated with a 21% lower risk of death from cancer, and the benefit was only apparent after 5 years of follow-up (44). The median duration of follow-up in our cohort was about 4 years. However, reduced breast cancer risk has been reported with aspirin usage as infrequent as once per week for 6 months or longer (16).

Our finding that VS-induced audiometric threshold shifts were the largest at high frequencies is consistent with reports that high-frequency sensorineural hearing loss is the most common type VS-induced hearing loss (40,45–47). Specifically, VS-induced high-frequency hearing loss is the dominant pattern in Danish (40), Egyptian (46), Turkish (45), and Korean (47) patients. The preponderance of high-frequency sensorineural hearing loss is thought to reflect mechanical compression of the tonotopically organized cochlear nerve by VS (48,49). However, audiometric threshold shifts due to sporadic VS do not correlate with the radiographic tumor size or tumor extent within the internal auditory canal (50,51), suggesting that hearing loss due to VS is multifactorial (52). We have recently demonstrated that VS secretions can cause direct cochlear damage (53,54), and that tumor necrosis factor alpha (TNFα) is an ototoxic molecule in VS secretions (53). Interestingly, TNFα induces NF-κB activation, while aspirin inhibits it (22,23).

Our current and previous (19,20) data motivate a prospective, randomized, placebo-controlled phase II trial of aspirin for VS treatment. Such a clinical trial would be an improvement over previous trials in terms of safety, treatment cost, and potential for widespread patient applicability. Previous clinical trials have often focused on expensive drugs and invasive procedures, which placed a heavy burden of care on both the provider and the patient. Our overarching objective has been to identify inexpensive drugs with lesser side effects than those caused by drugs that have been tested in clinical trials for VS thus far. Cytostatic drugs can be therapeutic without being cytotoxic because VSs are nonmalignant. Indeed, there has been a growing trend in simply observing nongrowing VSs (55), reserving surgery and radiation therapy for growing and symptomatic tumors. Our data suggests that aspirin may represent a noninvasive pharmacological approach to treatment that promises to slow and/or stop VS tumor growth. At best aspirin may prevent the need for surgery or radiation therapy; in the least aspirin may allow the patient and clinician more time to make decisions regarding timing of intervention. Previous research has indicated that inflammation may play a key role in VS development and progression, and histological analyses of resected tumors have found chronic inflammation, sheets of macrophages, nuclear atypia, and scarring (56). As chronic inflammation is a well-established trigger for tumor development (57) the systemic reduction of inflammation may provide potent and persistent benefits for the delaying tumor growth in VSs.

Limitations of our retrospective study include a relatively small sample size due to the inability to access serial MRI scans performed at outside institutions and reliance on self-reported aspirin use when precise dosing information was not available in medical records. By design, the study is correlative, not causative. It is possible that the overall health of patients on aspirin may be different from nonusers, which may independently affect VS growth. The role of cerebrovascular disease in VS progression has not been studied. Retrospective reporting of medication use is subject to error, particularly underreporting. However, recall bias is unlikely to explain our results because people with growing VSs would have to underreport usage more than people with stable VSs (19); patients with growing tumors are more likely to seek health care and provide detailed medical information. Careful assessment of all potential confounders will be required in a future prospective clinical trial to determine whether there is a role for aspirin in VS treatment.

CONCLUSION

Our findings, which align with the results of our original clinical study (19), with mechanistic studies in vitro (20), and with the known presence of pathologic immune response in VS (21), suggest a potential therapeutic role for aspirin in VS. A prospective, appropriately powered clinical study is needed to determine whether nonsteroidal antiinflammatory medications such as aspirin can prevent VS growth. The preponderance of VS-induced high-frequency sensorineural hearing loss in our population is consistent with that observed in other populations.

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REFERENCES

1. Pitts LH, Jackler RK, Fuller GN. Intracranial nerve sheath tumors. In: Levin VAE, editor. *Cancer in the Nervous System*. New York: Churchill Livingstone; 1996. pp. 199–210.
2. Stangerup SE, Caye-Thomasen P. Epidemiology and natural history of vestibular schwannomas. *Otolaryngol Clin North Am* 2012;45:257–68.
3. Evans DG, Howard E, Giblin C, et al. Birth incidence and prevalence of tumor-prone syndromes: Estimates from a UK family genetic register service. *Am J Med Genet A* 2010;152A:327–32.
4. Propp JM, McCarthy BJ, Davis FG, et al. Descriptive epidemiology of vestibular schwannomas. *Neuro Oncol* 2006;8:1–11.
5. Schmidt RF, Boghani Z, Choudhry OJ, et al. Incidental vestibular schwannomas: A review of prevalence, growth rate, and management challenges. *Neurosurg Focus* 2012;33:E4.
6. Anderson TD, Loevner LA, Bigelow DC, et al. Prevalence of unsuspected acoustic neuroma found by magnetic resonance imaging. *Otolaryngol Head Neck Surg* 2000;122:643–6.
7. Lin D, Hegarty JL, Fischbein NJ, et al. The prevalence of ''incidental'' acoustic neuroma. *Arch Otolaryngol Head Neck Surg* 2005;131:241–4.
8. Ohtani ITY, Omori K. Incidence of asymptomatic vestibular schwannoma and origin of the tumor in Japanese. *Otol Japan* 2007;17:615–20.
9. Matthies C, Samii M. Management of 1000 vestibular schwannomas (acoustic neuromas): Clinical presentation. *Neurosurgery* 1997;40:1–9.
10. Thomsen J, Zilstorff K, Tos M. Acoustic neuromas (diagnostic value of testing the function of the trigeminal nerve, the cerebellum and optokinetic nystagmus). *J Laryngol Otol* 1983;97:801–12.
11. Plotkin SR, Stemmer-Rachamimov AO, Barker FG 2nd, et al. Hearing improvement after bevacizumab in patients with neurofibromatosis type 2. *N Engl J Med* 2009;361:358–67.
12. Slusarz KM, Merker VL, Muzikansky A, et al. Long-term toxicity of bevacizumab therapy in neurofibromatosis 2 patients. *Cancer Chemother Pharmacol* 2014;73:1197–204.
13. Vane JR, Botting RM. The mechanism of action of aspirin. *Thromb Res* 2003;110:255–8.
14. Hong B, Krusche CA, Schwabe K, et al. Cyclooxygenase-2 supports tumor proliferation in vestibular schwannomas. *Neurosurgery* 2011;68:1112–7.
15. Rostom A, Dube C, Lewin G. *Use of Aspirin and NSAIDs to Prevent Colorectal Cancer*. Rockville, MD: Agency for Healthcare Research and Quality (US); 2007.
16. Terry MB, Gammon MD, Zhang FF, et al. Association of frequency and duration of aspirin use and hormone receptor status with breast cancer risk. *JAMA* 2004;291:2433–40.
17. Fink SP, Yamauchi M, Nishihara R, et al. Aspirin and the risk of colorectal cancer in relation to the expression of 15-hydroxyprostaglandin dehydrogenase (HPGD). *Sci Transl Med* 2014;6:233re2.
18. Tougeron D, Sha D, Manthravadi S, et al. Aspirin and colorectal cancer: Back to the future. *Clin Cancer Res* 2014;20:1087–94.
19. Kandathil CK, Dilwali S, Wu CC, et al. Aspirin intake correlates with halted growth of sporadic vestibular schwannoma in vivo. *Otol Neurotol* 2014;35:353–7.
20. Dilwali S, Kao SY, Fujita T, et al. Nonsteroidal anti-inflammatory medications are cytostatic against human vestibular schwannomas. *Transl Res* 2015;166:1–11.
21. Dilwali S, Briet MC, Kao SY, et al. Preclinical validation of anti-nuclear factor-kappa B therapy to inhibit human vestibular schwannoma growth. *Mol Oncol* 2015;9:1359–70.
22. Muller DN, Heissmeyer V, Dechend R, et al. Aspirin inhibits NF-kappaB and protects from angiotensin II-induced organ damage. *FASEB J* 2001;15:1822–4.
23. McCarty MF, Block KI. Preadministration of high-dose salicylates, suppressors of NF-kappaB activation, may increase the chemosensitivity of many cancers: An example of proapoptotic signal modulation therapy. *Integr Cancer Ther* 2006;5:252–68.
24. Niemczyk K, Vaneecloo FM, Lemaitre L, et al. The growth of acoustic neuromas in volumetric radiologic assessment. *Am J Otol* 1999;20:244–8.
25. Sorensen AG, Patel S, Harmath C, et al. Comparison of diameter and perimeter methods for tumor volume calculation. *J Clin Oncol* 2001;19:551–7.
26. Harris GJ, Plotkin SR, Maccollin M, et al. Three-dimensional volumetrics for tracking vestibular schwannoma growth in neurofibromatosis type II. *Neurosurgery* 2008;62:1314–9.
27. Walz PC, Bush ML, Robinett Z, et al. Three-dimensional segmented volumetric analysis of sporadic vestibular schwannomas: Comparison of segmented and linear measurements. *Otolaryngol Head Neck Surg* 2012;147:737–43.
28. Therasse P, Arbuck SG, Eisenhauer EA, et al. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. *J Natl Cancer Inst* 2000;92:205–16.
29. US Preventive Services Task Force. Summaries for patients. Aspirin for the prevention of cardiovascular disease: U.S. Preventive Services Task Force recommendation statement. *Ann Intern Med* 2009;150:I-37.
30. Niu NN, Niemierko A, Larvie M, et al. Pretreatment growth rate predicts radiation response in vestibular schwannomas. *Int J Radiat Oncol Biol Phys* 2014;89:113–9.
31. Vokurka EA, Herwadkar A, Thacker NA, et al. Using Bayesian tissue classification to improve the accuracy of vestibular schwannoma volume and growth measurement. *AJNR Am J Neuroradiol* 2002;23:459–67.
32. van de Langenberg R, de Bondt BJ, Nelemans PJ, et al. Follow-up assessment of vestibular schwannomas: Volume quantification versus two-dimensional measurements. *Neuroradiology* 2009;51:517–24.
33. Eisenhauer EA, Therasse P, Bogaerts J, et al. New response evaluation criteria in solid tumours: Revised RECIST guideline (version 1.1). *Eur J Cancer* 2009;45:228–47.
34. Varughese JK, Wentzel-Larsen T, Vassbotn F, et al. Analysis of vestibular schwannoma size in multiple dimensions: A comparative cohort study of different measurement techniques. *Clin Otolaryngol* 2010;35:97–103.
35. Kim YH, Kim DG, Han JH, et al. Hearing outcomes after stereotactic radiosurgery for unilateral intracanalicular vestibular schwannomas: Implication of transient volume expansion. *Int J Radiat Oncol Biol Phys* 2013;85:61–7.
36. West JS, Low JC, Stankovic KM. Revealing hearing loss: A survey of how people verbally disclose their hearing loss. *Ear Hear* 2016;37:194–205.
37. Diensthuber M, Lenarz T, Stover T. Determination of the clinical growth index in unilateral vestibular schwannoma. *Skull Base* 2006;16:31–8.
38. Opotowsky AR, McWilliams JM, Cannon CP. Gender differences in aspirin use among adults with coronary heart disease in the United States. *J Gen Intern Med* 2007;22:55–61.
39. Patel NB, Nieman CL, Redleaf M. Hearing in static unilateral vestibular schwannoma declines more than in the contralateral ear. *Ann Otol Rhinol Laryngol* 2015;124:490–4.
40. Thomsen J, Terkildsen K, Tos M. Acoustic neuromas. Progression of hearing impairment and function of the eighth cranial nerve. *Am J Otol* 1983;5:20–33.
41. Fayad JN, Semaan MT, Lin J, et al. Conservative management of vestibular schwannoma: Expectations based on the length of the observation period. *Otol Neurotol* 2014;35:1258–65.
42. Cerletti C, Bonati M, del Maschio A, et al. Plasma levels of salicylate and aspirin in healthy volunteers: Relevance to drug interaction on platelet function. *J Lab Clin Med* 1984;103:869–77.
43. Samlan SR, Jordan MT, Chan SB, et al. Tinnitus as a measure of salicylate toxicity in the overdose setting. *West J Emerg Med* 2008;9:146–9.
44. Garcia-Albeniz X, Chan AT. Aspirin for the prevention of colorectal cancer. *Best Pract Res Clin Gastroenterol* 2011;25:461–72.

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45. Tutar H, Duzlu M, Goksu N, et al. Audiological correlates of tumor parameters in acoustic neuromas. *Eur Arch Otorhinolaryngol* 2013;270:437–41.

46. Ragab A, Emara A, Shouker M, et al. Prospective evaluation of the clinical profile and referral pattern differences of vestibular schwannomas and other cerebellopontine angle tumors. *Otol Neurotol* 2012;33:863–70.

47. Lee SH, Choi SK, Lim YJ, et al. Otologic manifestations of acoustic neuroma. *Acta Otolaryngol* 2015;135:140–6.

48. *HFS*. Philadelphia, PAReference (48) is not available in PubMed. Please update as per style.: Lea & Febiger; 1993.

49. Sando I. The anatomical interrelationships of the cochlear nerve fibers. *Acta Otolaryngol (Stockh)* 1965;59:417–36.

50. Nadol JB Jr, Diamond PF, Thornton AR. Correlation of hearing loss and radiologic dimensions of vestibular schwannomas (acoustic Neuromas). *Am J Otol* 1996;17:312–6.

51. Caye-Thomasen P, Dethloff T, Hansen S, et al. Hearing in patients with intracanalicular vestibular schwannomas. *Audiol Neurootol* 2007;12:1–12.

52. Thakur JD, Banerjee AD, Khan IS, et al. An update on unilateral sporadic small vestibular schwannoma. *Neurosurg Focus* 2012;33:E1.

53. Dilwali S, Landegger LD, Soares VY, Deschler DG, Stankovic KM. Secreted factors from human vestibular schwannomas can cause cochlear damage. *Sci Rep* 2105;5:18599.

54. Soares VY, Atai NA, Fujita T, et al. Extracellular vesicles derived from human vestibular schwannomas associated with poor hearing damage cochlear cells. *Neuro Oncol* 2016. [Epub ahead of print].

55. Carlson ML, Habermann EB, Wagie AE, et al. The changing landscape of vestibular schwannoma management in the United States—a shift toward conservatism. *Otolaryngol Head Neck Surg* 2015;153:440–6.

56. Sobel RA. Vestibular (acoustic) schwannomas: Histologic features in neurofibromatosis 2 and in unilateral cases. *J Neuropathol Exp Neurol* 1993;52:106–13.

57. Lu H, Ouyang W, Huang C. Inflammation, a key event in cancer development. *Mol Cancer Res* 2006;4:221–33.

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